NOTE TO USERS

IDENTIFICATION OF CELL CYCLE REGULATORY PROTEINS THAT INTERACT WITH HCF-1

BY

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ABSTRACT

HCF-1, a transcriptional regulatory protein, was originally identified as an accessory factor for the induction of Herpes Simplex Virus immediate-early genes by the viral transactivator VP16. Recently, HCF-1 has emerged as a chromatin-binding transcriptional co-regulator that plays an essential role in cellular proliferation. In order to further characterize the function of HCF-1 and determine the mechanisms by which it contributes to cellular proliferation we have sought to identify novel HCF-1 interaction partners and elucidate their function. In this thesis, we describe the identification of key cell cycle regulatory proteins, Miz-1 and E2F-4, as novel HCF-1 interaction partners. Miz-1, a transcription factor that activates transcription of cell cycle inhibitory genes, is an integral part of the anti-mitogenic TGF-β pathway and contributes to cell cycle arrest following DNA damage and differentiation signals. HCF-1 associates with the Miz-1 transactivation domain and antagonizes Miz-1-dependent transcriptional activation of $p15^{INK4b}$, a cyclin-dependent kinase inhibitor, suggesting that HCF-1 can indirectly promote pRB family inactivation, and thus, cellular proliferation. E2F-4, a member of the E2F family of transcription factors that regulate cellular proliferation in conjunction with the retinoblastoma family of proteins, is involved in repression of E2F responsive genes at G0/G1 and consequently, cell cycle arrest or differentiation. Overexpression of E2F-4 suppresses HCF-1-mediated rescue of cellular proliferation, indicating an antagonistic role for these proteins in the cell cycle. Together, these findings contribute to identifying additional HCF-1 interaction partners and may provide insight into the molecular mechanism of HCF-1-mediated cell cycle progression.

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LIST OF ABBREVIATIONS

Ab	antibody
AD	activation domain
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
Ci	Curies
CDK	cyclin-dependent kinase
Da	Dalton
DBD	DNA binding domain
DHFR	dihydrofolate reductase
DNA	deoxyribonucleic acid
Fn3 repeats	fibronectin type 3 repeats
GABP	GA-binding protein
GST	glutathione S-transferase
HBM	HCF-binding motif
HCF-1	Host Cell Factor-1
HCF-2	Host Cell Factor-2
HCF_{PRO} repeats	HCF-1 proteolytic repeats
HDAC	histone deacetylase complex
HMT	histone methyltransferase
HPIP	HCF-1 β-propeller domain-interacting protein
HSV	Herpes Simplex Virus
IE genes	immediate-early genes
MCM proteins	mini-chromosome maintenance proteins
Miz-1	myc-interacting zinc finger protein
NES	nuclear export sequence
NLS	nuclear localization sequence
ORC	origin recognition complex
PGC-1	peroxisome proliferator-activated receptor γ co-activator-1
PEG	polyethylene glycol
POZ domain	Poxvirus and zinc finger protein domain
PP1	protein phosphatase 1
RNAase A	ribonuclease A
SAS	self-association sequence
SDS	sodium dodecyl sulphate
SnRNPs	small nuclear ribonucleoprotein particles
TK	thymidine kinase
VIC	VP16-induced complex
ZF	Zhangfei
2YT AMP	2YT media containing 100μg/mL ampicillin

CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW

Human host cell factor-1 (HCF-1) (also known as CFF, VCAF1, C1, and HCF) is a transcriptional regulatory protein that was originally identified as an accessory factor required for induction of Herpes Simplex Virus immediate-early genes in conjunction with the cellular octamer-binding transcription factor, Oct-1, and the viral transactivator VP16 (Gerster and Roeder 1988; Kristie *et al.*, 1989; Katan *et al.*, 1990; Xiao and Capone 1990; Herr, 1998). However, the function of HCF-1 in the uninfected cell is less well understood. It has recently become evident that HCF-1 is required for cellular proliferation and completion of cytokinesis (Goto *et al.*, 1997; Wilson *et al.*, 1997; Julien and Herr, 2003). The mammalian cell cycle is a finely regulated process, in which proteins that are involved in its regulation must be periodically expressed at specific points in the cycle (Lania *et al.*, 1999; Darnell, 2002). Temporal expression of specific growth-regulatory genes is often regulated at the level of transcription initiation through the formation of multiprotein-DNA complexes which influence transcription initiation (Lania *et al.*, 1999; Naar *et al.*, 2001; Darnell, 2002). Precise cell cycle control is essential for normal development and for the prevention of cancer, since oncogenesis may result from its disturbance (Malumbres and Barbacid, 2001). The work presented in this thesis sheds light on the mechanisms by which HCF-1 promotes cellular proliferation by describing novel protein-protein interactions and how these interactions may control expression of genes intimately involved in cell cycle control.

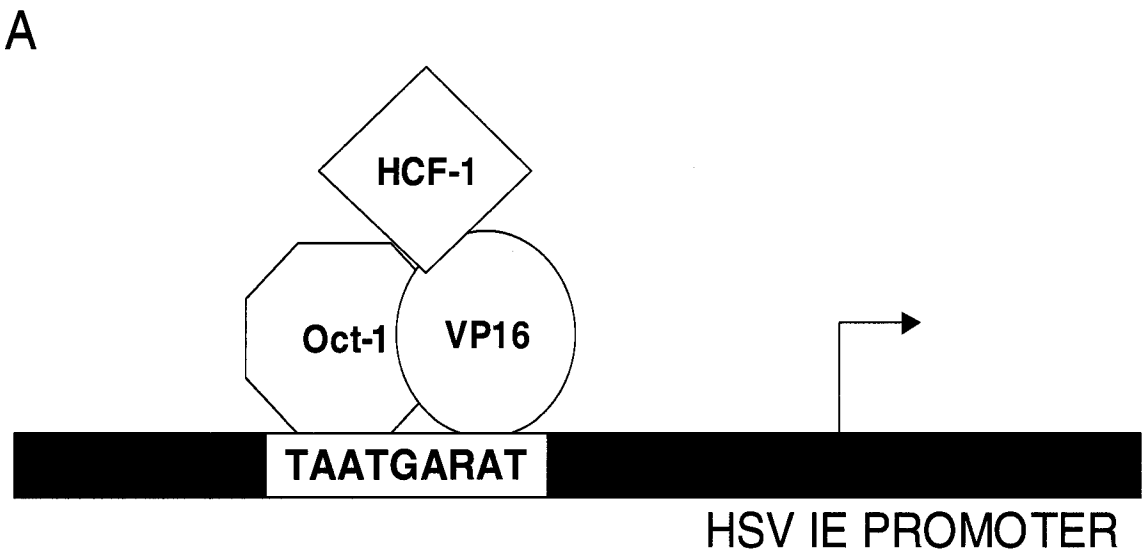
1.2 HISTORY OF HCF-1

The history of HCF-1 dates back nearly 20 years to investigations in a number of laboratories researching the mechanism of Herpes Simplex Virus (HSV) immediate-early (IE) gene transcription regulation. At the time, it was clear that a viral protein, VP16, was recruited to TAATGARAT elements upstream of immediate-early gene promoters in conjunction with a cellular protein, Oct-1 (O'Hare *et al.*, 1988; Stern *et al.*, 1989; Kristie and Sharp, 1990; Stern and Herr, 1991). However, efforts to form a VP16-induced complex on TAATGARAT elements *in vitro* with VP16 and Oct-1, in the absence of nuclear extracts, indicated that at least one other cellular factor must be required to form the VP16-induced complex and initiate expression of HSV immediate-early genes (Gerster and Roeder, 1988; Kristie *et al.*, 1989; Katan *et al.*, 1990; Xiao and Capone, 1990). This cellular protein was later cloned and named host cell factor, HCF (Kristie and Sharp, 1993; Wilson *et al.*, 1993; Kristie *et al.*, 1995). Thus, HCF-1 was first detected by virtue of its ability to stabilize the VP16-induced complex. HCF-1 has been conserved through metazoan evolution as extracts from insects and *C. elegans* can support VP16-induced complex formation on TAATGARAT elements (Kristie and Sharp, 1990; Liu *et al.*, 1999; Mahajan *et al.*, 2002b; Izeta *et al.*, 2003a).

The initiation of HSV-1 infection involves complex formation between viral (VP16) and cellular (HCF-1 and Oct-1) proteins (Herr, 1998). Upon infection, the viral transactivator VP16 (also known as αTIF, ICP25, Vmw65) is released into the cell to initiate a temporal cascade of viral gene expression producing α, β, and γ proteins; the three gene classes of HSV-1. VP16 stimulates the transcription of α (also known as

immediate-early, IE) genes through recognition of TAATGARAT target elements present in the upstream regions of IE genes (Triezenberg *et al.*, 1988a; Herr, 1998; Wysocka and Herr, 2003). The α genes are expressed immediately upon infection and the products of these genes are required for the synthesis of subsequent β and γ polypeptide groups resulting in viral DNA replication and capsid assembly (Ward and Roizman, 1994). VP16 has only weak intrinsic DNA binding activity and thus requires two cellular factors, the POU homeodomain protein Oct-1 and HCF-1, to efficiently bind to target elements and form a multiprotein complex termed the VP16-induced complex, VIC (Gerster and Roeder, 1988; O'Hare *et al.*, 1988; Stern *et al.*, 1989; Katan *et al.*, 1990; Xiao and Capone, 1990; Walker *et al.*, 1994; Cleary *et al.*, 1997). VP16, a 490 amino acid protein, contains two functionally distinct regions: an amino terminal region (amino acids 49-388) is responsible for VIC formation, while the carboxy-terminal 80 amino acids form a potent acidic activation domain that stimulates transcription (Triezenberg *et al.*, 1988b; Cousens *et al.*, 1989; Greaves and O'Hare, 1989; Greaves and O'Hare, 1990; Lai and Herr, 1997). Oct-1, a cellular transcription factor of 766 amino acids, is ubiquitously expressed and activates transcription of genes encoding small nuclear RNAs, snRNAs, which are involved in pre-mRNA splicing, histone H2B, and immunoglobin genes (Sturm *et al.*, 1988; Wysocka and Herr, 2003). It contains N- and C-terminal transactivation domains, enriched in glutamines or serines and threonines respectively, and a central DNA binding domain termed the POU domain (Sturm *et al.*, 1988; Sturm and Herr, 1988; Tanaka and Herr, 1990; Tanaka *et al.*, 1992).

VIC assembly requires sequence specific protein-DNA interactions as well as protein-protein interactions (see figure 1.1). Specifically, Oct-1 binds independently to TAATGARAT elements upstream of HSV IE genes providing DNA specificity to VIC formation (O'Hare *et al.*, 1988; Kristie and Sharp, 1990; Walker *et al.*, 1994; Cleary *et al.*, 1997), while VP16 is recruited to these sites through interaction with the Oct-1 POU homeodomain (Stern *et al.*, 1989; Kristie and Sharp, 1990; Stern and Herr, 1991). Interestingly, Oct-1 has been shown to have flexible DNA binding specificity and this has been exploited in its binding to TAATGARAT elements which may or may not contain an overlapping octamer binding sequence, ATGCAAAT (Walker *et al.*, 1994; Misra *et al.*, 1996; Cleary *et al.*, 1997). However, efficient recruitment of VP16 into the complex requires prior association with an additional cellular protein, HCF-1 to stabilize a conformation of VP16 which can more efficiently associate with Oct-1 (Wilson *et al.*, 1997). However, association of HCF-1 with VP16 is not in itself sufficient for VIC formation as two missense HCF-1 mutants, E102A and K105D are able to bind VP16 but prevent VIC formation (Simmen *et al.*, 1997). Upon complex formation, VP16 initiates IE gene transcription through a potent carboxy-terminal transactivation domain (Triezenberg *et al.*, 1988b; Cousens *et al.*, 1989) that interacts with basal transcription factors and a histone acetylase complex, which increases the rate of assembly of the pre-initiation complex (Ingles *et al.*, 1991; Lin *et al.*, 1991; Hall and Struhl, 2002). However, there is also evidence that both HCF-1 (Luciano and Wilson, 2002) and Oct-1 may contribute to this process (Wu *et al.*, 1994).



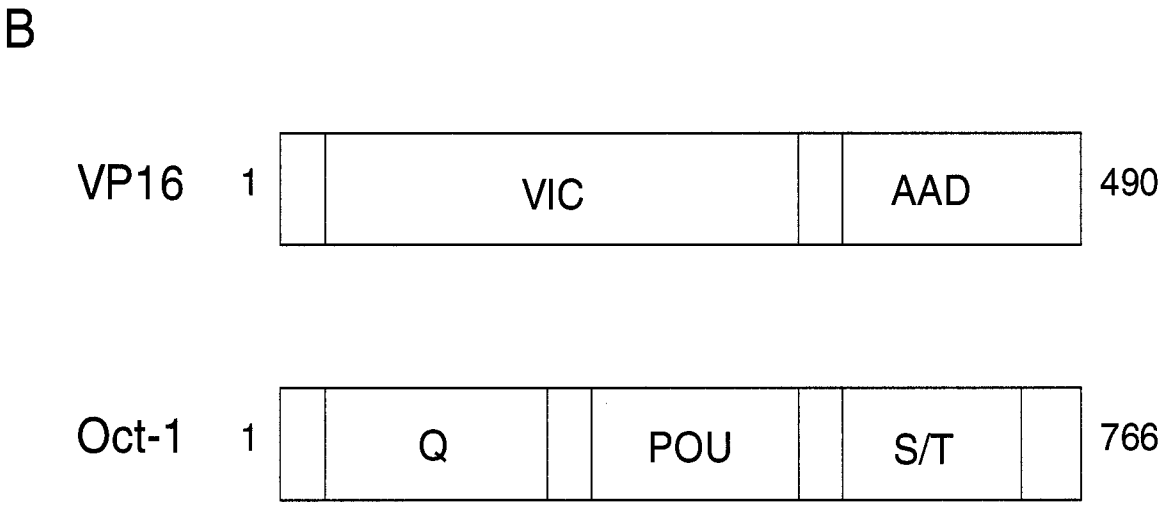


Figure 1.1: The expression of Herpes Simplex Virus Immediate-Early genes. A. HSV-1 immediate-early gene expression requires the assembly of a VP16-induced complex, VIC, consisting of viral, VP16, and cellular proteins, Oct-1 and HCF-1 on the TAATGARAT element found upstream of IE genes. B. Schematic diagrams of VP16 and Oct-1 indicate the modular domain structure of each. Abrreviations are as follows: VIC: VP16-induced complex; AAD: acidic activation domain; Q: glutamine-rich; S/T: serine/threonine-rich; POU: a highly charged 155-162 amino acid region of similarity contained in 3 mammalian transcription factors, Pit-1, Oct-1, Oct-2, and the product of the nematode gene unc-86 (Sturm *et al.*, 1988).

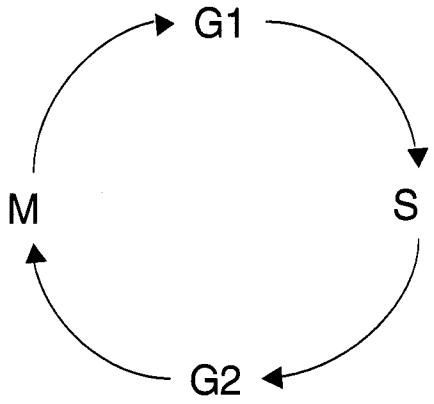
Although the function of HCF-1 has been well studied in the context of HSV immediate-early gene transcription its function in the uninfected cell has not been fully elucidated. In agreement with its function during HSV infection, HCF-1 does appear to function as a transcriptional co-activator for several of its interaction partners (Vogel and Kristie, 2000a; Lin *et al.*, 2002; Luciano and Wilson, 2002; Luciano and Wilson, 2003) although specific target genes have not been determined. Interestingly, HCF-1 appears to possess the ability to interact with transcriptional co-activators and co-repressors allowing for the possibility that HCF-1 may activate certain sets of genes while also repressing others (Wysocka and Herr, 2003). An initial clue into characterizing the cellular roles for HCF-1 was provided by the discovery of a hamster cell line that halts proliferation at its non-permissive temperature due to a missense mutation in HCF-1 (Goto *et al.*, 1997). Thus, in addition to its role in viral gene expression (Wysocka and Herr, 2003), HCF-1 is essential for normal cell cycle progression (Goto *et al.*, 1997; Wilson *et al.*, 1997; Julien and Herr, 2003). Although the precise functions of HCF-1 in the cell are unknown, HCF-1-mediated cellular proliferation may result, at least in part, via its regulation of gene transcription. The ability of HCF-1 to augment expression of growth-promoting genes, while also suppressing arrest-promoting genes may allow it to play a key role in cell cycle control where the temporal expression of growth-regulating genes is often regulated at the level of transcription initiation.

1.3 CELL CYCLE CONTROL

The cell cycle can be divided into 4 distinct stages. The S, or synthesis, phase is the stage in which the DNA is replicated, while the M, or mitotic, phase is the stage in

which the cellular contents are divided. G1 and G2 are transient gap phases that precede the S and M phases of growing cells, respectively (Trimarchi and Lees, 2002). G0, often viewed together with G1 as G0/G1, represents a substate of G1 in which cells deprived of growth factors exit the cycle and enter into a long-term quiescent state from which they can be reactivated by growth factors to re-enter the cycle. Orderly progression through these phases is controlled by the sequential activation of cyclin-dependent kinases, CDKs, which regulate the retinoblastoma protein and related pocket proteins by phosphorylation (see figure 1.2; Dyson, 1998; Harbour and Dean, 2000; Malumbres and Barbacid, 2001; Tonini *et al.*, 2002). Pocket proteins bind to E2F transcription factors when hypophosphorylated, and prevent activation of growth-promoting genes during the G1 phase of the cell cycle (Hiebert *et al.*, 1992; Helin *et al.*, 1993; Dyson, 1998). E2F was originally defined as a cellular factor required for the adenovirus early region 1A (E1A) transforming protein to mediate the transcriptional activation of the viral E2 promoter (Nevins, 1992). In fact, E2F activity arises from a family of heterodimeric transcription factors where each heterodimer consists of one member of the E2F family bound to a member of the DP family (DeGregori, 2002; Trimarchi and Lees, 2002). Subsequent studies have shown that the E2F family of transcription factors plays a central role in regulating cellular proliferation by controlling the expression of genes required for cell cycle progression, which include enzymes involved in nucleotide biosynthesis (ex. DHFR, and TK), cell cyle regulatory proteins (ex. Cyclin A, D1, E, Cdc2, and Cdc25A) and proteins essential for DNA synthesis itself (ex. DNA Pol, Cdc6, ORC1, and MCM proteins) (DeGregori, 2002; Trimarchi and Lees, 2002). Binding of

A



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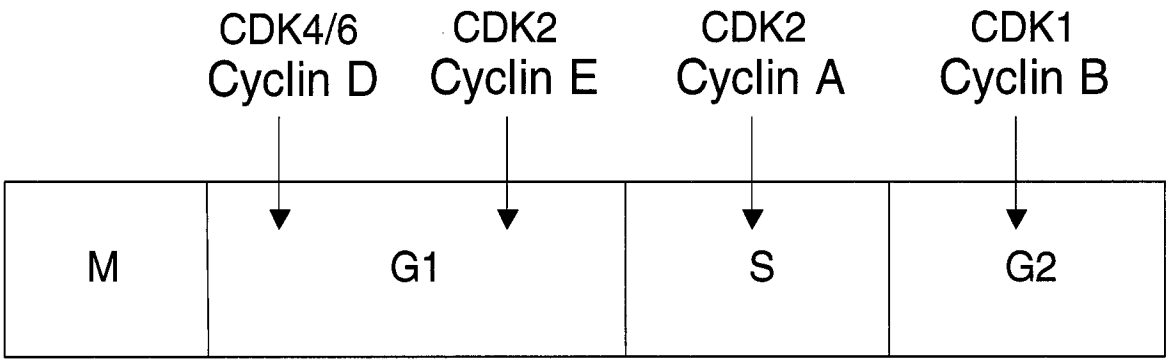

Figure 1.2: The mammalian cell cycle. A. Schematic representation of cell cycle phases. The cell cycle can be divided into four distinct stages. G1 and G2 are gap phases that occur before the S (synthesis) and M (mitosis) phases, respectively. B. Orderly progression through the cell cycle phases is controlled by the sequential activation of cyclin-dependent kinases, CDKs. Cell cycle progression may be halted by the activation of checkpoint pathways that ultimately lead to the inactivation of one or more CDK-cyclin complex(es).

pRb tumour suppressor family (Rb, p107, p130) proteins, also referred to as 'pocket proteins', to E2F factors inhibit their ability to activate transcription, and in some cases, converts E2F factors from activators to repressors of transcription (Hiebert *et al.*, 1992; Weintraub *et al.*, 1992; Helin *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Vairo *et al.*, 1995; Frolov *et al.*, 2004). Regulation by pRB and pocket protein family members is controlled through their phosphorylation status, via mitogenic growth factors that induce the sequential activation of cyclin-dependent kinase complexes which phosphorylate pRb, or conversely via growth-inhibitory signals which inhibit cyclin-dependent kinase activity, thus preventing pocket protein phosphorylation and cellular proliferation (Dyson, 1998; Harbour and Dean, 2000; Stevaux and Dyson, 2002; Trimarchi and Lees, 2002). In G0 and early G1, pocket proteins are hypophosphorylated, interact with E2Fs through the E2F transactivation domain, and prevent transcriptional activation by E2Fs or actively represses transcription by recruiting HDAC complexes to promoters (Chellappan *et al.*, 1991; Beijersbergen *et al.*, 1995; Mayol *et al.*, 1995; Hansen *et al.*, 2001; Stevaux and Dyson, 2002). In mid G1, as a result of activation of cyclin-dependent kinases, pocket proteins are phosphorylated, first by cyclinD-CDK4/6 and then by cyclinE-CDK2 (Dyson, 1998; Malumbres and Barbacid, 2001), resulting in dissociation of pocket proteins from E2Fs. This leads to nuclear export of E2F-4, while E2F-1-3, now free of pocket proteins in late G1, promote increased expression of growth-promoting genes at the correct time (Lindeman *et al.*, 1997; Takahashi *et al.*, 2002; Gaubatz *et al.*, 2001). Thus, upon pocket protein phosphorylation by CDKs, E2F is

released from the inhibitory complexes, allowing transcriptional activation of growth-promoting genes in mid-late G1 (Dyson, 1998).

The central players controlling pocket protein phosphorylation status are the CDKs; serine/threonine kinases that form active heterodimeric complexes upon binding to cyclins, their regulatory subunits (Lees, 1995; Malumbres and Barbacid, 2001). CDK4/6 form active complexes with the D-type cyclins and initiate pRb phosphorylation in mid-G1, whereas CDK2 is activated by the E-type cyclins and is required to complete G1 and initiate S phase by phosphorylating pRb on additional residues (Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). CyclinA- and B-dependent CDKs, activated later during the cell division cycle maintain pRb in a hyperphosphorylated form until cells exit mitosis and pRb is returned to a hypophosphorylated state in the next G1 phase (Sherr and Roberts, 1999).

In addition to cyclin binding, a variety of other mechanisms contribute to regulate the activity of cyclin-dependent kinases both positively and negatively (see figure 1.3; Lees, 1995; Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). In fact, regulation of CDK activity requires, in addition to cyclin binding, regulation of its phosphorylation status, and complex formation with proteins termed cyclin-dependent kinase inhibitors, CKIs (Lees, 1995; Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). Regulation by phosphorylation can be both a positive or negative regulatory control depending on the residues phosphorylated (Malumbres and Barbacid, 2001). For instance, CDKs must be phosphorylated on a threonine residue, located in their T loop, for proper catalytic activity, by a CDK7-cyclinH complex known as CAK, CDK-

activating kinase (Malumbres and Barbacid, 2001). In contrast, phosphorylation of adjacent threonine and tyrosine residues on CDKs, mediated by dual specificity kinases such as WEE1 and MYT1, results in their inhibition since phosphorylation of these residues sterically hinders substrate access to the catalytic site (Lees, 1995; Malumbres and Barbacid, 2001). Conversely, this inhibition can be relieved by CDC25 phosphatases, which dephosphorylate these residues and trigger mitosis (Lees, 1995, Malumbres and Barbacid, 2001). Interaction with proteins termed CKIs will also inactivate CDKs (Lees, 1995; Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). CKIs have been postulated to play critical roles in growth arrest and cell differentiation in response to growth-inhibitory signals transduced from the extracellular environment (Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). CKIs are of two types, the INK4 family and the WAF1/KIP family (Nakayama and Nakayama, 1998; Sherr and Roberts, 1999; Ortega *et al.*, 2002). The four members of the INK4 family, $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$, exert their inhibitory activity by binding to the CDK4/6 kinases and preventing their association with D-type cyclins (Sherr and Roberts, 1999; Ortega *et al.*, 2002). The three members of the WAF1/KIP family, $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, form complexes with both CDK4/6-cyclinD and CDK2-cyclinE complexes (Nakayama and Nakayama, 1998; Sherr and Roberts, 1999). However, they only inhibit the kinase activity of CDK2-cyclinE complexes by binding within the catalytic subunit, thereby dismantling its ATP-binding site (Sandhu *et al.*, 1997; Sherr and Roberts, 1999). CDK4/6-cyclinD complexes can bind WAF1/KIP inhibitors without losing kinase activity and therefore, titrate these inhibitors from CDK2/cyckinE (Sherr and Roberts,

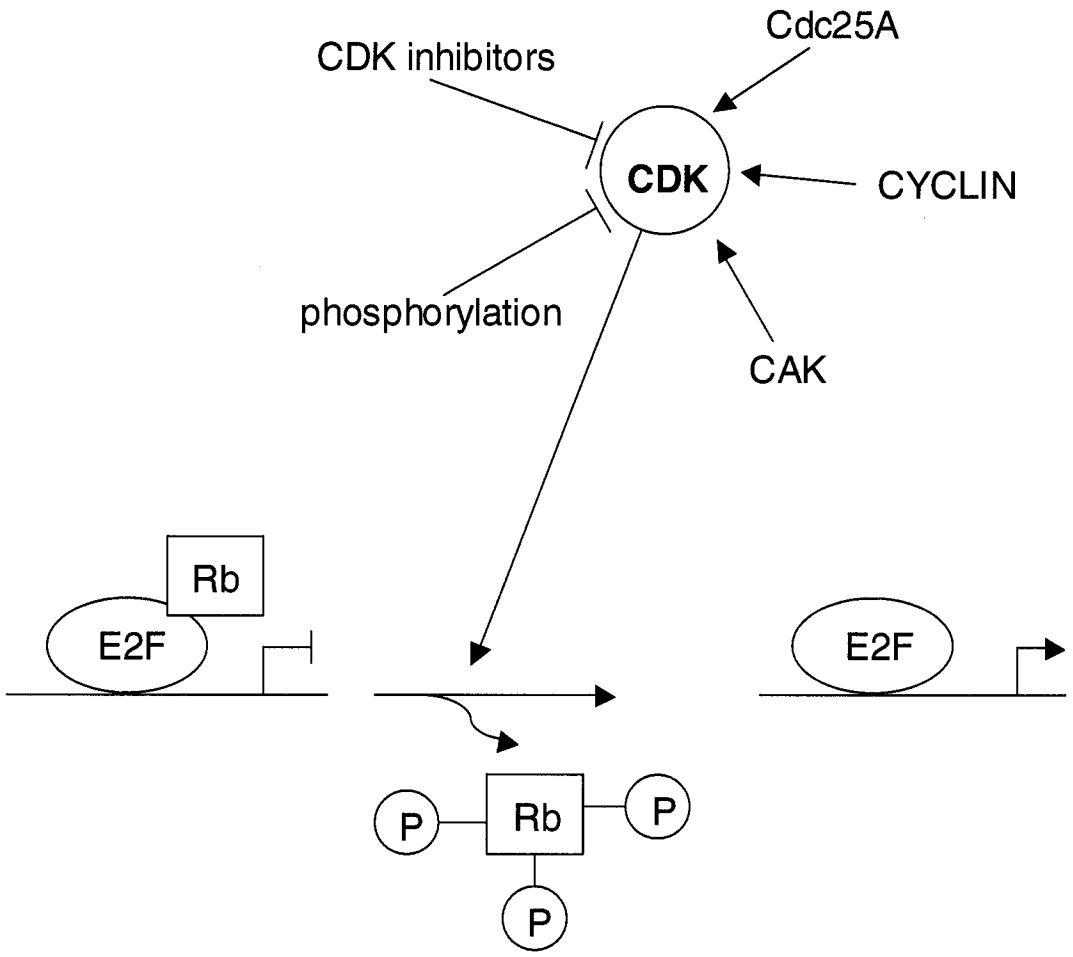

Figure 1.3: CDKs control the phosphorylation status of Rb proteins. Cell cycle control is a highly regulated process that is influenced by both positive and negative growth-regulatory signals. These signals ultimately impinge on cyclin-dependent kinases, CDKs, that are negatively regulated by binding of proteins known as CDK inhibitors and some phosphorylation events. Conversely, CDKs can be activated by removing inhibitory phosphates, controlled by the Cdc25A phosphatase, adding activating phosphates, controlled by CDK-activating kinase, CAK, and through binding cyclins. Activated CDKs phosphorylate Rb in G1, releasing 'free E2F' allowing transcriptional activation of growth-promoting genes including cell cycle regulatory proteins and enzymes involved in DNA synthesis.

1999). Thus, expression of INK4 proteins ensures cell cycle arrest by binding to CDK4/6-cyclinD complexes and inhibiting their kinase activity, while also liberating bound WAF1/KIP proteins, and thereby indirectly inhibiting CDK2-cyclinE complexes (Reynisdottir *et al.*, 1995; Sandhu *et al.*, 1997; Sherr and Roberts, 1999).

1.4 TRANSCRIPTIONAL CONTROL

To ensure precise cell cycle control, proteins that are involved in its regulation must be periodically expressed at specific phases in the cycle. The expression of such growth regulatory proteins is largely regulated at the transcriptional level during specific cell cycle phases by a diverse set of DNA sequence-specific transcription factors and an assortment of co-regulatory factors that bridge the sequence-specific transcription factors to the transcriptional machinery to drive the formation of active transcription initiation complexes (Lemon and Tjian, 2000; Woychik and Hampsey, 2002; Smale and Kadonaga, 2003). This requires the interplay of a multi-protein RNA polymerase II (Pol II) complex and associated general transcription factors (TFIIA, -B, -D, -E, -F, and –H), which are required for promoter recognition and the catalysis of RNA synthesis, in addition to a large number of transcription factors, multiple core promoter elements, and chromatin remodeling and modifying factors (see figure 1.4; Naar *et al.*, 2001; Woychik and Hampsey, 2002; Smale and Kadonaga, 2003; Taatjes et al., 2004).

The formation of active transcription initiation complexes involves the formation of a multi-protein complex at the transcriptional start site. This process may follow a stepwise assembly mechanism whereby initially a complex is formed between TFIID, TFIIA, and TFIIB capable of recognizing and binding to the TATA promoter element.

Subsequently, the recruitment of hypophosphorylated RNA Pol II and TFIIF to this multi-protein-DNA complex forms a stable closed complex. Finally, an activated open complex is formed by the addition of TFIIE and TFIIH, which stimulate an ATP-dependent promoter melting event, followed by promoter clearance and nascent RNA synthesis upon hyperphosphorylation of the RNA Pol II C-terminal domain (Lemon and Tjian, 2000; Woychik and Hampsey, 2002). In contrast to the stepwise assembly model for transcriptional initiation, another model envisions the targeted recruitment of a completely pre-assembled RNA Pol II holoenzyme to the transcriptional start site (Lemon and Tjian, 2000).

The cascade of events that precede the activation of transcription must eventually lead to the basal transcriptional machinery at the core promoter (Butler and Kadonaga, 2002; Levine and Tjian, 2003; Smale and Kadonaga, 2003). In order to initiate promoter-dependent transcription or respond to transcriptional regulatory proteins, the core RNA polymerase II enzyme that transcribes protein-coding genes was found to require several additional factors, termed general transcription factors, GTFs (Goodrich *et al.*, 1996; Butler and Kadonaga, 2002; Smale and Kadonaga, 2003). TFIID is a general transcription factor composed of the TATA Box binding protein, TBP, and several TBP-associated factors, TAFs, and has emerged as one of the central components of the transcriptional apparatus (Albright and Tjian, 2000; Woychik and Hampsey, 2002). TFIID functions to recognize the core promoter and provide a surface upon which the other general transcription factors and RNA Pol II can assemble (Naar *et al.*, 2001; Smale and Hampsey, 2002; Woychik and Hampsey, 2002). Furthermore, individual TAFs can

recognize specific core promoter elements such as the initiator element, Inr, and the downstream promoter element, DPE, in conjuction with TBP binding to the TATA element to define the start site of transcription (Naar *et al.*, 2001; Smale and Kadonaga, 2003). TAFs also play an important role in transcriptional activation by providing various co-activator interfaces targeted by different activation domains of sequence-specific transcription factors and co-activators (Hochheimer and Tjian, 2003).

Although the general transcription factors are sufficient for accurate initiation by RNA Pol II, transcription stimulation requires the action of sequence-specific transcription factors that bind to cognate DNA elements and interact with general transcription factors or TAFs and serve to recruit the multi-protein RNA Pol II complex to the core promoter, and a variety of multi-protein complexes termed co-regulators (Lemon and Tjian, 2000; Naar *et al.*, 2001). These co-regulators can be divided into two broad categories: (1) those proteins which interact with, or are themselves components of, the RNA polymerase II transcriptional machinery and (2) chromatin modifiers (Naar *et al.*, 2001; Levine and Tjian, 2003). Co-regulators that interact with components of the RNA polymerase II transcriptional machinery are not sequence-specific transcription factors, rather they affect transcriptional outcomes by bridging the sequence-specific transcription factors to the multi-protein RNA polymerase II complex (Goodrich *et al.*, 1996; Albright and Tjian, 2000; Naar *et al.*, 2001; Levine and Tjian, 2003). Chromatin modifiers can be further sub-divided into 2 classes: ATP-dependent nucleosome remodeling complexes and histone modifiers. These proteins may be directly recruited by sequence-specific transcription factors or in some cases may be recruited

independently to chromatin through histone interactions (Naar *et al.*, 2001). ATP-dependent nucleosome remodeling complexes utilize the energy derived from the hydrolysis of ATP to help sequence-specific transcription factors and the transcriptional apparatus gain access to their cognate DNA sequences by reorganizing repressive chromatin structures by displacing histone-DNA contacts (Naar *et al.*, 2001; Levine and Tjian, 2003). In addition, chromatin structure may be modified through histone acetylation or deacetylation by histone acetlytransferases, HATs, and histone deacetylase complexes, HDACs respectively (Naar *et al.*, 2001; Levine and Tjian, 2003). Actively transcribed genes have been correlated with increased histone acetylation, which counter repressive effects of local chromatin structure, whereas these effects are countered by the actions of HDACs which serve to suppress transcriptional activation of silenced genes (Naar *et al.*, 2001; Levine and Tjian, 2003). Thus, these chromatin-modifying complexes affect chromatin structure to regulate transcriptional outcomes.

Additionally, a variety of covalent histone modifications, including acetylation, phosphorylation, methylation, and ubiquitination provide recruitment sites for interaction with other proteins that are involved in transcriptional regulation (Berger, 2002; Featherstone, 2002). These modifications represent an additional level of combinatorial control since the pairs of modifications and the sequence of alterations determine whether the outcome is active or repressed transcription (Berger, 2002). An additional level of complexity is also provided by histone acetyltransferases, which in addition to modifying histones, also acetylate other target proteins, such as sequence-specific transcription

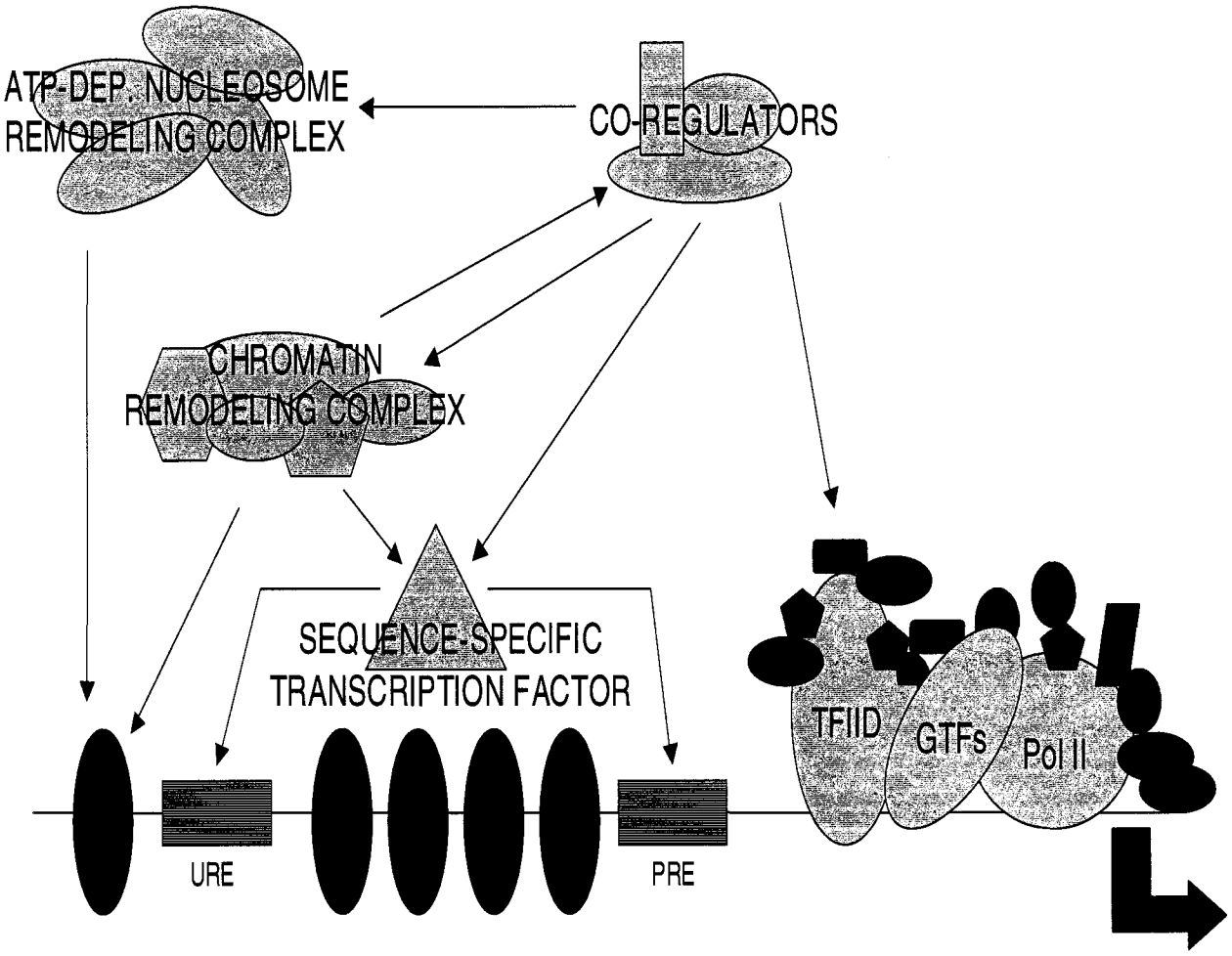

Figure 1.4: A variety of activities displayed by distinct classes of proteins are involved in regulating gene transcription. Regulated transcription initiation requires the interplay of several classes of multi-subunit complexes that include the RNA polymerase II (Pol II) complex and associated general transcription factors, GTFs, sequence-specific transcription factors and co-regulators that collectively bind to their cognate response elements in the DNA sequence, labeled here generically as upstream response element, URE, and proximal response element, PRE, and various chromatin-remodeling and –modifying complexes which act on nucleosomes, depicted here as dark ovals. Dark shapes decorating TFIID, GTFs, and Pol II represent TAFs and other associated polypeptides which comprise these multi-subunit complexes. Together, these activities serve to drive the formation of active transcription initiation complexes. This figure has been adapted from Hochheimer and Tjian, 2003 and Naar *et al.*, 2001.

factors or components of the transcriptional apparatus to alter their activities to regulate transcriptional outcomes (Naar *et al.*, 2001).

Thus, transcriptional control mechanisms in eukaryotic organisms operate with elaborate complexity including diverse sequence-specific transcription factors and a hostof co-regulatory complexes that collectively serve to remodel chromatin structure to facilitate activator DNA binding and recruit additional regulatory proteins, and ultimately recruit the RNA polymerase II transcriptional machinery to specific target genes at specific times, such as specific points in the cell cycle to allow for controlled and orderly progression through the cycle.

1.5 HCF-1 STRUCTURAL ELEMENTS

HCF-1 is synthesized as a 2035 amino acid protein of approximately 300 kD that is autocatalytically processed following transport to the nucleus, at a series of six 26 amino acid repeat elements termed HCF_{PRO} repeats located near the middle of the HCF-1 precursor polypeptide (Kristie and Sharp, 1993; Wilson *et al.*, 1993; Kristie *et al.*, 1995; Wilson *et al.*, 1995a; Vogel and Kristie, 2000b). Cleavage occurs at a glutamate residue, PPCE/THET, within the HCF_{PRO} repeat to generate N- and C-terminal fragments which range in size from approximately 110 kD to 150 kD, that remain non-covalently associated through 2 pairs of self-association sequences located in each fragment (Wilson *et al.*, 1995a; LaBoissiere *et al.*, 1997; Hughes *et al.*, 1999; Wilson *et al.*, 2000). The SAS1 (self-association sequence 1) pair has been mapped to residues 360-402 (SAS1N) and 1812-2002 (SAS1C) while the SAS2 (self-association sequence 2) pair have been mapped to residues 491-755 (SAS2N) and 1436-1756 (SAS2C) (Wilson *et al.*, 2000).

In addition to the HCF_{PRO} repeats and self-association sequences, HCF-1 possesses several additional modular domains (see figure 1.5a). Amino acids 20-380 contain six repeated copies of a kelch-like sequence first identified in the *Drosophila* protein Kelch, proposed to form four-stranded β sheets that assemble into a barrel-like six bladed β-propeller (Wilson *et al.*, 1997; Adams *et al.*, 2000). This domain, termed the HCF kelch domain (also referred to as the VIC, VP16-induced complex, domain) is sufficient to interact with VP16, stabilize the VP16-induced complex on IE gene promoters, and promote transcription (Simmen *et al.*, 1997; Wilson *et al.*, 1997; Lee and Herr, 2001). In addition to subserving a role in HSV IE gene expression, the kelch domain is variously involved in HCF-1-mediated cell cycle progression (Goto *et al.*, 1997), pre-mRNA processing (Ajuh *et al.*, 2002), chromatin association (Wysocka *et al.*, 2001), and recruiting histone methyltransferase enzymes which modify chromatin structure (Wysocka *et al.*, 2003). The kelch domain appears to function as a protein-protein interaction module (Adams *et al.*, 2000). Not surprisingly, it has been found to mediate interaction between HCF-1 and a variety of proteins (see figure 1.5a). Interestingly, these interaction partners all share a common feature; the presence of a tetrapeptide motif, D/EHxY, now referred to as the HCF-binding motif, HBM. However, interactions between the kelch domain and the HBM are made exquisitely sensitive through variations in the kelch domain and corresponding variations in the residues flanking the HBM. The kelch domain assembles into a six bladed β-propeller with loops that run between the adjacent β-strands of one blade or link adjacent blades that protrude from the flattened β-propeller structure imparting specific protein-protein interaction sites

(Wilson *et al.*, 1997; Adams *et al.*, 2000). Although the overall tertiary structure is conserved, the primary sequence identity of the kelch domain is modest expecially within the loops, allowing for specific protein interactions (Adams *et al.*, 2000). Conversely, the non-conserved sequences flanking the HBM contribute significantly to the interaction with HCF-1 (Wu *et al.*, 1994; Lai and Herr, 1997; Mahajan and Wilson, 2000). This is supported by the fact that mutation of valine 358 to alanine in VP16, which is positioned three residues upstream of the HBM (Wu *et al.*, 1994), as well as mutation of residues 385-387 in VP16, which are downstream of the HBM (Lai and Herr, 1997) contribute to interaction with HCF-1. Thus, the interaction between the HCF-1 kelch domain and HBM-containing partners is based upon the three dimensional β-propeller structure and HBM, however an additional level of sensitivity is provided through variations in the loops of the β-propeller and corresponding variations in the residues flanking the HBM. This situation is similar to interactions between co-activators and nuclear hormone receptors mediated by the LxxLL motif found in co-activators, where selectivity is determined not solely by the LxxLL motif but also the sequence of the flanking residues (Coulthard *et al.*, 2003).

Downstream of the HCF kelch domain lies a region enriched in basic amino acids, termed the basic domain, which is required for HCF-1-mediated cellular proliferation (Wilson *et al.*, 1997), and interacts with a variety of cellular transcription factors (figure 1.5a; Wysocka and Herr, 2003) and the Sin3 protein which is part of a histone deacetylase complex (Wysocka *et al.*, 2003). The carboxy-terminal subunit of the HCF-1

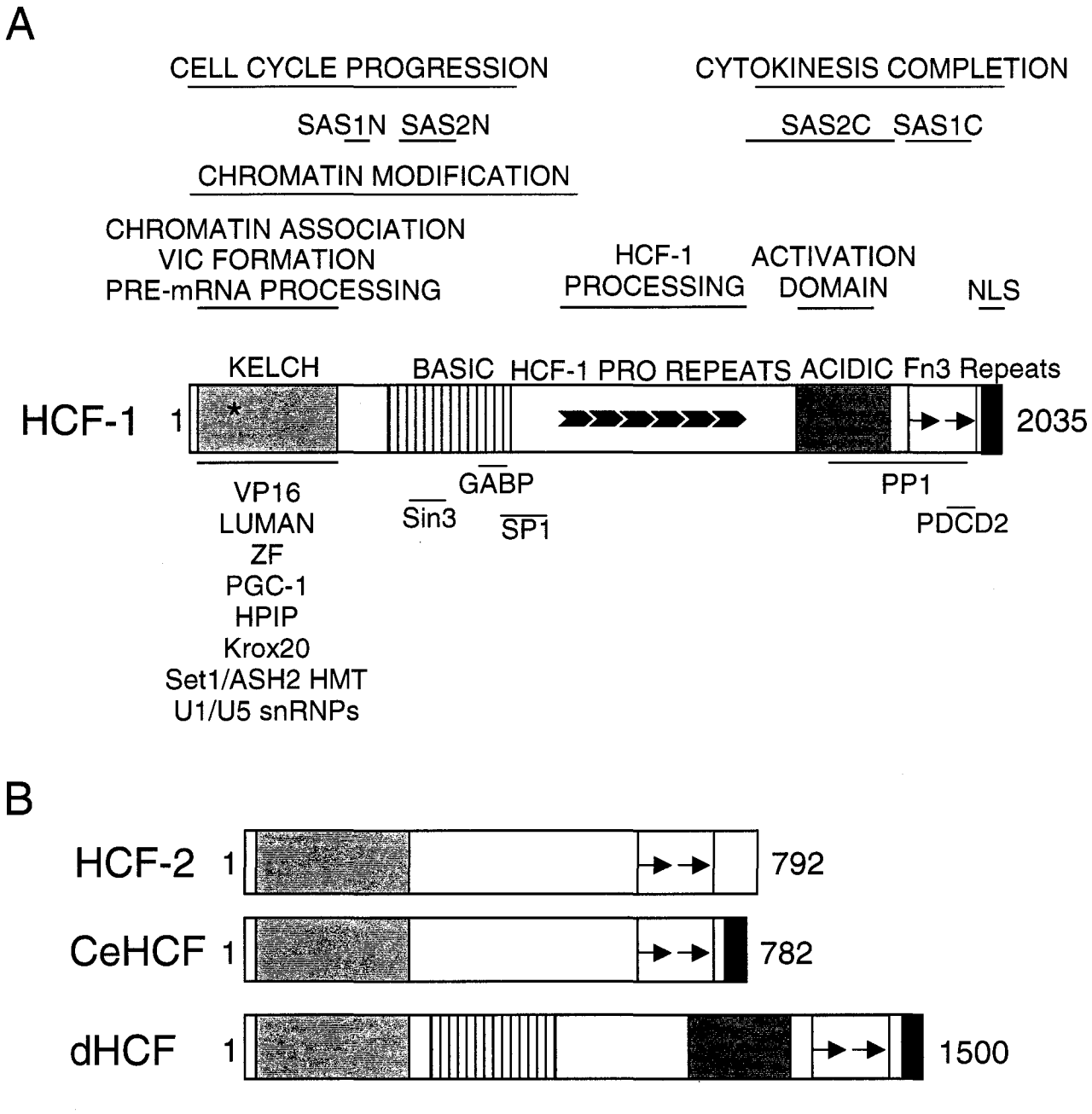

Figure 1.5: Schematic representation of HCF-1 and its related proteins. A. The modular domain structure of HCF-1 is shown including the various functions and interaction partners associated with each structural domain. The asterisk indicates the position of the P134S mutation that causes temperature-sensitive cellular proliferation arrest in tsBN67 cells. B. The modular domain structure of HCF-1 related proteins, HCF-2, *Caenorhabditis elegans* (CeHCF), and *Drosophila melanogaster* (dHCF), are shown with conserved domains between HCF proteins indicated by the same shading or pattern as in HCF-1.

polypeptide contains an acidic domain, followed by two fibronectin type 3 (Fn3) repeats and a nuclear localization sequence, NLS.

Downstream of the HCF-1_{PRO} repeats lies a region enriched in acidic residues termed the acidic domain. This domain has been shown to function as an autonomous transcriptional activation domain and co-expression of the transcriptional co-activator, p300, enhances transactivation by the HCF-1 acidic activation domain suggesting that it functions through recruitment of p300 (Luciano and Wilson, 2002). Importantly, the HCF-1 acidic activation domain cooperates with VP16 to augment viral immediate-early gene expression and improve viral titres (Luciano and Wilson, 2002). Furthermore, the HCF-1 acidic activation domain is required for efficient transactivation by its cellular interaction partners, Luman and Krox20 (Luciano and Wilson, 2002; Luciano and Wilson, 2003). Downstream of the acidic domain, the protein contains two fibronectin type 3 repeats that make up one of the self-association sequences (SAS1C) that tether the N- and C-terminal fragments following proteolytic cleavage (Wilson *et al.*, 2000). Structurally, Fn3 repeats fold into two β sheets packed against each other to form an extended groove that can form a docking site for interacting proteins (Campbell and Spitzfaden, 1994). At the extreme carboxy-terminus of the protein is located a nuclear localization sequence that directs subcellular localization of HCF-1 (LaBoissiere *et al.*, 1999). The NLS is a bipartite sequence composed of two basic residues, a spacer of any ten amino acids, and a cluster of basic amino acids in which 3/5 must be basic (LaBoissiere *et al.*, 1999). This sequence is responsible for the nuclear localization of HCF-1, and also provides a mechanism for the nuclear import of associated proteins such

as VP16 (LaBoissiere *et al.*, 1999). Although HCF-1 is predominantly a chromatin-associated nuclear protein (LaBoissiere *et al.*, 1999; Wysocka *et al.*, 2001), HCF-1 is not detected in the nucleus of sensory neurons, where the HSV virus establishes latency (Kristie *et al.*, 1999). Interestingly, reactivation of the virus in mouse model systems results in nuclear localization of HCF-1 indicating that HCF-1 subcellular localization is a critical determinant in the viral lytic/latent cycle (Kristie *et al.*, 1999). Further evidence for a dynamic localization pattern for HCF-1 came with the identification of an HCF-1 interacting protein called HPIP which contains a leucine-rich nuclear export sequence and serves to shuttle HCF-1 out of the nucleus and into the cytosol (Mahajan *et al.*, 2002a). Finally, in primary G0 cells, a 50 kD N-terminal fragment of HCF-1 arises by proteolysis, which can be found in the cytosol (Scarr *et al.*, 2000). Therefore, although HCF-1 contains a *bona fide* NLS, and is predominantly bound to chromatin in the nucleus, certain tissues or phases of the cell cycle may contain cytosolic HCF-1.

1.6 CELLULAR ROLES FOR HCF-1

In addition to its role in viral gene expression (Wysocka and Herr, 2003), HCF-1 is essential for normal cell cycle progression (Goto *et al.*, 1997; Wilson *et al.*, 1997; Julien and Herr, 2003). This fact became evident with the discovery of the hamster temperature-sensitive cell line (tsBN67) that arrests cell cycle reversibly at G0/G1 at the non-permissive temperature (Goto *et al.*, 1997). Analysis of this cell line revealed a temperature-sensitive mutation in HCF-1 in which amino acid 134, which is located within the kelch domain, is mutated from proline to serine, encoded by the single X chromosome-linked allele of the HCF-1 gene (Goto *et al.*, 1997). Importantly, this

function is not unique to tsBN67 cells, as siRNA depletion of HCF-1 was sufficient to cause cell cycle arrest in normal and oncogenically transformed cells (Julien and Herr, 2003). The arrested cells contain active hypophosphorylated pRb (Reilly and Herr, 2002) and the majority have a 2C-DNA content consistent with a G0/G1-phase arrest (Goto *et al.*, 1997; Reilly and Herr, 2002). However, a minority of cells contain active hypophosphorylated pRb, and arrest with a 4C-DNA content (Goto *et al.*, 1997) and are binucleated, consistent with a G0/G1 arrest after improper cytokinesis (Reilly and Herr, 2002). Thus, both cell cycle arrest and a cytokinesis defect are due to loss of HCF-1 function. The functional importance of HCF-1 processing had long been enigmatic but has recently been shown to be required for completion of cytokinesis (Julien and Herr, 2003).

The identification of the P134S mutation causing the tsBN67 phenotype reveals the importance of the kelch domain in cell cycle progression by HCF-1 (Goto *et al.*, 1997; Wilson *et al.*, 1997). Interestingly, the P134S mutation in HCF-1, which results in cell cycle arrest, abrogates binding to VP16 (Wilson *et al.*, 1997) foretelling the existence of cellular factors that mimic VP16 in their interaction with HCF-1. However, the kelch domain alone is not sufficient for cell cycle progression since rescue of the tsBN67 cell cycle progression defect required both the kelch domain and the adjacent basic domain (Wilson *et al.*, 1997), suggesting cooperativity between the two domains in ensuring cellular proliferation. HCF-1-mediated cellular proliferation is thus likely the product of interactions with a multitude of cellular factors that target both the kelch and basic domains. The importance of the basic region in cellular proliferation is underscored by

recent studies with the HCF-1 family member HCF-2 and the related *Caenorabiditis elegans* CeHCF and *Drosophila melanogaster* dHCF homologues. HCF-1, HCF-2, CeHCF and dHCF share conserved amino- and carboxy-terminal domains, however they lack significant sequence identity with the basic region and the central HCF_{PRO} repeats (Johnson *et al.*, 1999; Liu *et al.*, 1999; Lee and Herr, 2000; Izeta *et al.*, 2003a). Thus, while HCF-2, CeHCF, and dHCF are able to support VP16-induced complex formation (Liu *et al.*, 1999; Lee and Herr, 2002; Mahajan *et al.*, 2002b), these proteins are unable to rescue the temperature-sensitive cell cycle defect in tsBN67 cells (Johnson *et al.*, 1999; Lee and Herr, 2002; Izeta *et al.*, 2003a). In accordance with the basic domain contributing to HCF-1-mediated cellular proliferation, various proteins that have a function in cell cycle control have been shown to interact with the basic domain, such as the transcription factors GABP (Vogel and Kristie, 2000a), Sp1 (Gunther *et al.*, 2000), and Sin3 HDAC (Wysocka *et al.*, 2003). Interestingly, evidence suggests that HCF-1 regulates proliferation by opposing pRb family function. Specifically, research has shown that inactivation of the pRb family with SV40 large T antigen and adenovirus E1A can rescue the tsBN67 cell proliferation defect and cytokinesis defect (Reilly *et al.*, 2002). However, HCF-1-mediated cell cycle progression most likely requires more than pRb family inactivation since it was recently shown that although HeLa cells lack functional pRb family proteins due to the presence of the human papilloma virus E7 protein, depletion of HCF-1 results in cell cycle arrest (Julien and Herr, 2003).

In addition to cellular proliferation, the N-terminal kelch domain is necessary and sufficient for chromatin association (Wysocka *et al.*, 2001). At the non-permissive

temperature, the P134S mutation causes dissociation of HCF-1 from chromatin prior to cell cycle arrest, suggesting that loss of HCF-1 chromatin association contributes to the tsBN67 cell proliferation defect (Wysocka *et al.*, 2001). Furthermore, the kelch domain has recently been shown to interact with splicing snRNP complexes (Ajuh *et al.*, 2002). HCF-1 is a component of the spliceosome complex and is required for splicing (Ajuh *et al.*, 2002). At the non-permissive temperature the P134S mutation inhibits HCF-1's interaction with splicing snRNPs, causing inefficient spliceosome assembly and inhibiting splicing suggesting that HCF-1-dependent splicing events may also be required for cellular proliferation (Ajuh *et al.*, 2002).

Although the precise molecular roles of HCF-1 in the cell are unknown, HCF-1-mediated cell cycle progression results, at least in part, via regulation of gene transcription. HCF-1 possesses the characteristics of a general transcriptional co-regulatory factor (Wysocka and Herr, 2003). Through its amino-terminal kelch domain it is thought to indirectly interact with chromatin through association with other DNA binding transcription factors (Wysocka *et al.*, 2001). Furthermore, through interactions with histone methyltransferases (Set1/ASH2 HMT) and histone deacetylases (Sin3 HDAC) (Wysocka *et al.*, 2003), which are involved in chromatin modification, and a variety of transcription factors, HCF-1 has been shown to modulate transcription directly (Lu *et al.*, 1998; Luciano and Wilson, 2000; Vogel and Kristie, 2000a; Lin *et al.*, 2002; Luciano and Wilson, 2002; Luciano and Wilson, 2003). Moreover, HCF-1 has been shown to possess an intrinsic transactivation domain that augments viral and cellular gene

transcription in association with its interaction partners (Luciano and Wilson, 2002; Luciano and Wilson, 2003).

1.7 CELLULAR HCF-1 INTERACTION PARTNERS

The large size and modular domain structure of HCF-1 provides many potential protein-protein interaction interfaces. Figure 1.5a illustrates the variety of interactions and the domains of HCF-1 involved in these interactions. HCF-1 has been found to interact with a variety of cellular proteins including DNA-binding transcription factors, transcriptional co-regulatory proteins, and protein- and chromatin-modifying factors. The following is a review of the known HCF-1 interaction partners that may provide insights into the mechanisms of actions of HCF-1-mediated cellular proliferation. In addition to binding the viral protein, VP16 (Simmen *et al.*, 1997; Wilson *et al.*, 1997), the amino-terminal kelch domain interacts with the following cellular proteins; Luman (also known as LZIP) (Freiman and Herr, 1997; Lu *et al.*, 1997), Zhangfei (ZF) (Lu and Misra, 2000a), PGC-1 (Lin *et al.*, 2002), Krox20 (Luciano and Wilson, 2003), HPIP (Mahajan *et al.*, 2002a), Set1/ASH2 HMT (Wysocka *et al.*, 2003), and the U1/U5 snRNPs (Ajuh *et al.*, 2002). These are a diverse set of proteins with various functions that share a common feature that targets them to the HCF-1 kelch domain. First identified in VP16, these proteins all share a tetrapeptide motif, D/EHxY, termed the HBM, which targets the kelch domain (Hayes and O'Hare, 1993; Lai and Herr, 1997; Simmen *et al.*, 1997).

1.7.1 Luman (LZIP)

Luman is a 371 amino acid, ubiquitously expressed cyclic AMP response element (CRE)-binding protein/activating transcription factor 1 protein of the basic leucine zipper

superfamily (Freiman and Herr, 1997; Lu *et al.*, 1997). In addition to a basic DNA binding domain, and a leucine zipper motif, Luman contains an amino terminal transactivation domain comprised of two LxxLL motifs, a motif known to mediate interaction with nuclear hormone receptors and found in a number of transcriptional co-activators (Heery *et al.*, 1997; Torchia *et al.*, 1997; Rosenfeld and Glass, 2001) and the HBM (Lu *et al.*, 1998; Luciano and Wilson, 2000). Mutation of the HBM in Luman abrogates binding to HCF-1 and reduces transactivation by Luman, indicating a co-activator function for HCF-1 (Luciano and Wilson, 2000). Conversely, the HCF-1 tsBN67 cell proliferation mutant (P134S) fails to interact with Luman (Freiman and Herr, 1997; Lu *et al.*, 1998). However, a more extensive mutational analysis of the HCF-1 kelch domain demonstrated that binding of Luman was neither necessary nor sufficient for HCF-1-mediated cellular proliferation (Mahajan and Wilson, 2000). Luman contains a hydrophobic segment in its carboxy-terminus that functions as an endoplasmic reticulum (ER) transmembrane domain (Lu and Misra, 2000b) and anchors its N-terminus in the cytosol, which is regulated by regulated intramembrane proteolysis (Raggo *et al.*, 2002). In cells overexpressing Luman, HCF-1 was found in the cytosol and these cells are resistant to HSV-1 infection (Lu and Misra, 2000).

1.7.2 Zhangfei

Zhangfei is a 272 amino acid protein, structurally similar to Luman with a basic domain-leucine zipper region, an amino terminal acidic activation domain, and an HCF-binding motif in its C-terminus (Lu and Misra, 2000a). Mutation of the HBM in Zhangfei abrogates binding to HCF-1 and conversely the HCF-1 tsBN67 cell

proliferation mutant (P134S) fails to interact with Zhangfei (Lu and Misra, 2000a). Interestingly, ectopically expressed Zhangfei prevented the expression of several HSV proteins in cells infected with the virus (Lu and Misra, 2000a).

1.7.3 PGC-1

PGC-1 (peroxisome proliferator-activated receptor γ co-activator-1) is a transcriptional co-activator for a variety of nuclear hormone receptors, nuclear respiratory factors, and muscle-specific transcription factors that plays a critical role in regulating multiple aspects of energy metabolism including adaptive thermogenesis, fatty acid β-oxidation, hepatic gluconeogenesis, glucose uptake and drives the formation of slow twitch fibres in skeletal muscle (Puigserver and Spiegelman, 2003). PGC-1α and PGC-1β are distinct genes encoding proteins of conserved sequence (Puigserver and Spiegelman, 2003). PGC-1α (797 amino acids) and PGC-1β (1014 amino acids), in addition to containing an HBM (Lin *et al.*, 2002), also contain LxxLL motifs that are responsible for ligand-dependent interaction with nuclear hormone receptors (Rosenfeld and Glass, 2001; Puigserver and Spiegelman, 2003). They function as transcriptional co-activators and contain an N-terminal acidic activation domain as well as a C-terminal RNA-binding motif (Puigserver and Spiegelman, 2003). Consistent with the presence of an RNA binding motif, PGC-1α has been found in a complex with splicing factors suggesting that this factor can participate in the RNA splicing process (Monsalve *et al.*, 2000). Sequence analysis of PGC-1α and PGC-1β identified the presence of an HCF-binding motif in these proteins (Lin *et al.*, 2002). Subsequent analysis determined that the HCF-1 kelch domain binds to both PGC-1α and PGC-1β (Lin *et al.*, 2002) and

augments their transcriptional activity, indicating a co-activator function for HCF-1 (Lin *et al.*, 2002).

1.7.4 HPIP

HPIP is a broadly expressed 138 amino acid HCF-1 kelch domain binding protein that contains an HCF-binding motif as well as a leucine-rich nuclear export sequence (Mahajan *et al.*, 2002a). HPIP shuttles between the nucleus and cytosol and leads to an accumulation of HCF-1 in the cytosol which may serve to regulate its function in HSV-1-infected cells and/or the uninfected cell (Mahajan *et al.*, 2002a).

1.7.5 KROX20

Krox20, a 470 amino acid protein, is a member of the zinc finger transcription factor family (Chavrier *et al.*, 1988a; Chavrier *et al.*, 1988b). Originally identified as a member of the serum response immediate-early gene family (Chavrier *et al.*, 1988a), subsequent studies have revealed that it is required for normal hindbrain development and myelination of the peripheral nervous system (Schneider-Maunoury *et al.*, 1993; Swiatek and Gridley, 1993; Schneider-Maunoury *et al.*, 1997; Nagarajan *et al.*, 2001). Krox20 was found to interact with HCF-1 using a database search for cellular transcription factors containing the HBM (Luciano and Wilson, 2003). Krox20 contains three zinc fingers in its C-terminus responsible for DNA binding to GC-rich regions and an N-terminal acidic activation domain, within which the HBM is embedded (Vesque and Charnay, 1992; Luciano and Wilson, 2003). Mutation of the HBM diminishes transactivation by Krox20 and abrogates binding to HCF-1 indicating that HCF-1 functions as a co-activator for Krox20 with a contribution from the HCF-1 activation

domain (Luciano and Wilson, 2003). Thus, in addition to a role in cellular proliferation, HCF-1 may co-operate with Krox20 in development.

1.7.6 U1/U5snRNPs

HCF-1 was found to co-localize with nuclear structures that contain pre-mRNA splicing factors and to physically interact with complexes containing splicing snRNPs (Ajuh *et al.*, 2002). Furthermore, HCF-1 was found to be a component of the spliceosome complex and required for splicing (Ajuh *et al.*, 2002). The HCF-1 tsBN67 cell proliferation mutant (P134S), at the non-permissive temperature, inhibited HCF-1's interaction with U1 and U5 splicing snRNPs, causing inefficient spliceosome assembly and inhibiting splicing, suggesting that HCF-1-dependent splicing events may be required for cell cycle progression (Ajuh *et al.*, 2002).

1.7.7 Set1/ASH2 HMT and Sin3 HDAC

Consistent with a function for HCF-1 as a general transcriptional co-regulatory factor, recent results indicate that HCF-1 interacts with two distinct protein complexes with histone methyltransferase and histone deacetylase activity (Wysocka *et al.*, 2003). The kelch domain interacts with the HBM-containing trithorax-related Set1/ASH2 histone methyltransferase (HMT), generally associated with activation of transcription, while the basic domain associates with the Sin3 histone deacetylase (HDAC) complex, generally associated with repression of transcription (Wysocka *et al.*, 2003). Interestingly, HCF-1 was found in a large complex, tethering HMT and HDAC complexes together even though they are associated with opposing transcriptional outcomes (Wysocka *et al.*, 2003). However it appears that the interactions are context-

dependent since HCF-1 molecules bound to VP16 are selectively bound to the activating histone methyltransferase complex in the absence of the repressive histone deacetylase complex, consistent with its role in activating transcription in this context (Wysocka *et al.*, 2003). This mechanism may be generally used by HCF-1 in its interaction with a variety of transcription factors, since it has been shown to effect both transcriptional co-activation and co-repression (Lu *et al.*, 1998; Luciano and Wilson, 2000; Vogel and Kristie, 2000; Lin *et al.*, 2002; Luciano and Wilson 2002; Luciano and Wilson, 2003).

In addition to the Sin3 histone deacetylase complex (Wysocka *et al.*, 2003), the HCF-1 basic domain interacts with two cellular transcription factors: GABP (Vogel and Kristie, 2000a) and Sp1 (Gunther *et al.*, 2000).

1.7.8 GABP

GABP, a member of the ETS domain transcription factor family (Watanabe *et al.*, 1993; Batchelor *et al.*, 1998), is a heterodimeric factor consisting of a DNA-binding subunit (α subunit) and a transactivation subunit (β subunit) (Watanabe *et al.*, 1993; Sawa *et al.*, 1996; Batchelor *et al.*, 1998). In addition, the β subunit exists in two forms, GABPβ1 and GABPβ2, that differ in their C-termini (Watanabe *et al.*, 1993; de la Brousse *et al.*, 1994). β1 contains an additional leucine zipper which allows it to homodimerize in addition to heterodimerization with the α subunit, to form heterotetramers, while β2 does not and only forms heterodimers with the α subunit (Watanabe *et al.*, 1993; de la Brousse *et al.*, 1994). HCF-1 was found to interact with both forms of the β subunit (Vogel and Kristie, 2000a). HCF-1 targets the transactivation domain within the β subunit and mutations that reduce GABP transactivation also reduce

HCF-1 binding, indicating that HCF-1 functions as a co-activator for GABP-mediated transcription activation (Sawa *et al.*, 1996; Vogel and Kristie, 2000a). Previous studies have shown that GABP binds to sequences adjacent to the TAATGARAT elements in HSV-1 IE gene promoters (Triezenberg *et al.*, 1988a; LaMarco and Knight, 1989; Douville *et al.*, 1995) and that mutation of these elements decrease VP16-dependent transactivation, suggesting that GABP plays a role in transcription of HSV-1 IE genes (Triezenberg *et al.*, 1988a; Wu *et al.*, 1994). It is anticipated that HCF-1 may assemble GABP into the core enhancer complex along with the VIC complex for synergistic enhancement of IE gene expression.

1.7.9 Sp1

Sp1 is a ubiquitously expressed transcription factor that contains a C-terminal zinc finger DNA binding domain that binds to GC-rich sequences called Sp1 sites, and N-terminal transactivation domains composed of glutamine-rich and serine/threonine-rich stretches (Philipsen and Suske, 1999). Sp1 has been implicated in the activation of a large number of genes and is involved in a diverse set of cellular processes including cell cycle regulation (Philipsen and Suske, 1999; Black *et al.*, 2001). Interestingly, Sp1 sites have been found in the promoters of cyclin-dependent kinase inhibitor genes and Sp1 has been shown to activate the expression of $p15^{INK4b}$ and $p21^{Cip1}$ (Li *et al.*, 1995; Feng *et al.*, 2000; Pagliuca *et al.*, 2000; Pardali *et al.*, 2000). HCF-1 was found to interact with Sp1 through a yeast 2-hybrid screen using the Sp1 N-terminal transactivation domains as bait (Gunther *et al.*, 2000), however, the functional significance of this interaction remains to be determined.

The C-terminal fragment of HCF-1, which is required for proper completion of cytokinesis (Julien and Herr, 2003), has been shown to interact with two cellular proteins, PP1 (Ajuh *et al.*, 2000) and PDCD2 (Scarr and Sharp, 2002).

1.7.10 PP1

A screen with a digoxygenin-labelled protein phosphatase 1 (PP1) probe identified HCF-1 as a PP1-interacting protein (Ajuh *et al.*, 2000). PP1 is a member of the family of serine/threonine phosphatases and has been shown to be involved in the regulation of several cellular processes including cell division, pre-mRNA processing, and progression and exit from mitosis in mammalian cells (Cohen, 2002). Several proteins have been shown to bind and regulate PP1 activity (Cohen, 2002). Indeed, HCF-1 was shown to inhibit the activity of PP1 although what role this interaction has is currently undefined (Ajuh *et al.*, 2000).

1.7.11 PDCD2

PDCD2 is a ubiquitously expressed 344 amino acid protein, highly homologous to Rp8, a rat gene associated with apoptosis (Kawakami *et al.*, 1995). A yeast 2-hybrid screen using the HCF-1 C-terminal domain as bait identified PDCD2 as an HCF-1 interacting protein (Scarr and Sharp, 2002). PDCD2 was shown to interact with the NCoR/Sin3A corepressor complex and suppress HCF-1-mediated proliferation, indicating antagonistic roles for HCF-1 and PDCD2 in cell cycle regulation (Scarr and Sharp, 2002). Whether PDCD2 prevents HCF-1-mediated proliferation through repression of HCF-1-directed transcription of growth promoting genes is an intriguing possibility that awaits formal proof.

1.8 HCF-1 RELATED PROTEINS

HCF-1 related proteins have thus far been characterized from *Caenorhabditis elegans* (CeHCF), *Drosophila melanogaster* (dHCF), and humans (HCF-2). As shown in figure 1.5b, HCF-2 and CeHCF share the amino-terminal kelch domain and the carboxy-terminal Fn3 repeats with HCF-1, but lack the intervening sequences including the HCF-1_{PRO} repeats, which are necessary for proteolytic cleavage, and the basic domain which is necessary for cellular proliferation in HCF-1 (Johnson *et al.*, 1999; Liu *et al.*, 1999). Although HCF-2 and CeHCF are able to support VP16-induced complex formation (Liu *et al.*, 1999; Lee and Herr, 2001), consistent with the lack of these two regions, neither HCF-2 nor CeHCF is proteolytically processed or able to rescue the temperature-sensitive cell cycle defect in tsBN67 cells (Johnson *et al.*, 1999; Lee and Herr, 2001). In fact, co-expression of HCF-2 inhibits rescue by HCF-1, suggesting that the two factors share a common interaction partner(s) (Johnson *et al.*, 1999). Unlike HCF-1, CeHCF and HCF-2 lack HCF_{PRO} repeats and are not proteolytically processed (Johnson *et al.*, 1999; Liu *et al.*, 1999). Therefore, it is surprising to find that the SAS1 elements of HCF-1 are conserved in HCF-2 and CeHCF (Wilson *et al.*, 2000). Herr and co-workers (Wilson *et al.*, 2000) suggested that the presence of SAS1 sequences in CeHCF and HCF-2 may indicate a dual role for these sequences in HCF-1 function that is conserved in CeHCF and HCF-2. Thus, it was anticipated that these sequences (SAS1) are conserved in CeHCF and HCF-2, for protein-protein interactions with shared cellular factors.

In contrast to HCF-2 and CeHCF, dHCF contains more regions of similarity to HCF-1 (Mahajan *et al.*, 2002b; Izeta *et al.*, 2003a). In addition to the kelch domain,

which supports dHCF-mediated VP16-induced complex formation, and Fn3 domains, dHCF contains corresponding regions enriched in basic and acidic residues (Mahajan *et al.*, 2002b; Izeta *et al.*, 2003a). However, while these regions are enriched in basic and acidic residues, they do not display significant sequence identity with the corresponding HCF-1 regions (Izeta *et al.*, 2003a). Although dHCF contains a region enriched in basic residues this region most likely lacks functions of the HCF-1 basic region since it cannot substitute for HCF-1 to rescue the temperature-sensitive cell cycle defect in tsBN67 cells (Izeta *et al.*, 2003a).

dHCF, like CeHCF and HCF-2, lacks the HCF_{PRO} repeats and therefore would not be expected to undergo proteolytic processing (Mahajan *et al.*, 2002b; Izeta *et al.*, 2003a). Consistent with the lack of HCF_{PRO} repeats in the primary sequence of dHCF, O'Hare and colleagues found overexpressed dHCF in BHK cells to be unprocessed (Izeta *et al.*, 2003a). In contrast, Wilson and colleagues found overexpressed dHCF in drosophila SL2 cells to undergo proteolytic cleavage at one or more sites within the central region to produce N- and C-terminal fragments which remain non-covalently associated, as with HCF-1 (Mahajan *et al.*, 2002b).

In terms of subcellular localization, dHCF and CeHCF are more similar to HCF-1 than HCF-2 (Lee and Herr, 2001; Izeta *et al.*, 2003a). Like HCF-1, CeHCF and dHCF contain a conserved NLS at their carboxy-termini which is responsible for their nuclear localization (Liu *et al.*, 1999; Lee and Herr, 2001; Izeta *et al.*, 2003a). In contrast, HCF-2 lacks a conserved NLS and localizes to both the nucleus and cytosol in unsynchronized cells (Johnson *et al.*, 1999; Lee and Herr, 2001). Interestingly, a recent report by O'Hare

and colleagues demonstrates that CeHCF contains a dual targeting signal that can target the protein to the mitochondria, in addition to the nucleus, in mammalian cells due to interdigitated determinants located within the CeHCF C-terminus (Izeta *et al.*, 2003b). However, the physiological relevance of this function to this family of proteins remains to be determined.

1.9 OBJECTIVES OF RESEARCH AND KEY FINDINGS

HCF-1 is a transcriptional regulatory protein that was originally identified as an accessory factor for the induction of Herpes Simplex Virus immediate-early genes by the viral transactivator VP16, and has recently emerged as a chromatin-binding transcriptional co-regulator that plays key roles in cellular proliferation, cytokinesis, and pre-mRNA processing (Wysocka and Herr, 2003). A role for HCF-1 in cell cycle regulation has been shown through the finding of a temperature-sensitive hamster cell line, tsBN67, which undergoes cell cycle arrest at G0/G1 at the non-permissive temperature due to a single amino acid mutation in which proline-134 is changed to serine (Goto *et al.*, 1997). As shown in figure 1.5a, HCF-1 is a large protein that contains a variety of modular domains. HCF-1-mediated cellular proliferation requires both the N-terminal kelch domain as well as the adjacent basic domain (Wilson *et al.*, 1997). In order to determine how these domains contribute to cellular proliferation we have sought to identify novel protein-protein interactions for these domains.

Interaction partners were sought via two distinct approaches. In one approach we utilized the yeast 2-hybrid screen (Fields and Song, 1989) to search for kelch- and basic domain-interacting partners for HCF-1. Although a screen with the kelch domain only

yielded previously identified HCF-1-interaction partners (ie. Luman and Zhangfei), a screen performed with a fragment of HCF-1 which included the basic domain as bait ($HCF\text{-}1_{450\text{-}1439}$) yielded Miz-1, and a previously uncharacterized protein, we have termed HIP-2, as a novel HCF-1 interaction partners. Miz-1, originally identified through 2-hybrid screening for c-myc-interacting proteins (Peukert *et al.*, 1997), is an integral part of the anti-mitogenic TGF-β signaling pathway and also contributes to cell cycle arrest following DNA damage and differentiation signals (Seoane *et al.*, 2001; Herold *et al.*, 2002; Seoane *et al.*, 2002; Wu *et al.*, 2003; Zhao *et al.*, 2004).

We have demonstrated that Miz-1 targets the HCF-1 basic domain, and conversely, HCF-1 associates with the Miz-1 N-terminal POZ domain in addition to a C-terminal domain which also functions as a transcriptional activation domain. HCF-1 interaction with Miz-1 was shown to prevent recruitment of the transcriptional co-activator, p300, and consequently repress Miz-1 activation of $p15^{INK4b}$ gene transcription, a cyclin-dependent kinase inhibitor, which suggests that HCF-1 can indirectly promote pRB family inactivation by phosphorylation, and hence, cellular proliferation. However, Miz-1 expression did not prevent HCF-1-mediated rescue of the temperature-sensitive cellular proliferation defect in tsBN67 cells.

In addition to Miz-1, the HCF-1 basic domain has been shown to associate with GABP (Vogel and Kristie, 2000a). We sought to characterize its interaction with the HCF-1 basic domain and determine its contribution to HCF-1-mediated cellular proliferation. To this end, we have confirmed this interaction and describe a triple alanine mutation within the HCF-1 basic domain which impairs binding to GABP.

Interestingly, this HCF-1 mutant is also impaired in its ability to rescue the temperature-sensitive cellular proliferation defect in tsBN67 cells. In addition, we have utilized HCF-2, a human HCF-1-like protein that antagonizes HCF-1-mediated cellular proliferation, to provide additional insights into the role played by basic domain interacting partners in HCF-1-mediated cellular proliferation. We demonstrate that HCF-2 associates with both Miz-1 and GABP, and that a small carboxy-terminal fragment of HCF-2 which selectively interacts with GABP mimics the ability of full length HCF-2 to prevent HCF-1-mediated rescue of the temperature-sensitive defect in tsBN67 cells. Together, these results suggest that the HCF-1/GABP interaction may be required for HCF-1-mediated cellular proliferation.

The second approach taken to identify kelch domain-interaction partners exploited previous studies that have shown that the interaction between HCF-1 and VP16 and several cellular proteins is mediated by a tetrapeptide motif, D/EHxY, in the interaction partners termed the HCF-binding motif (Wysocka and Herr, 2003). Thus, in order to identify potential HCF-1 kelch domain interaction partners, we conducted database searches for HBM-containing cellular proteins. This approach has generated a large set of putative HCF-1 interaction partners, of which we have shown that two members of the E2F transcription factor family, E2F-1 and E2F-4, contain an HBM and interact with HCF-1. The E2F transcription factor family is a key regulator of cell cycle control which in conjunction with the retinoblastoma protein family determines whether a cell will proliferate, differentiate, or undergo apoptosis.

We have focused on the HCF-1/E2F-4 interaction and demonstrate that E2F-4 contains a functional HBM and binds HCF-1 through two domains: the kelch and basic domains. Furthermore, a mutation in the E2F-4 HBM abrogates binding to the kelch domain but not the basic domain, implying that the HBM contacts the kelch domain, as expected, and a second region of E2F-4 contacts the basic domain. As such, the P134S mutation in the context of the kelch domain abrogated binding to E2F-4. Finally, a biological consequence of this interaction was demonstrated when it was shown that expression of E2F-4 prevents HCF-1-mediated rescue of tsBN67 cell cycle progression.

Thus, we have identified Miz-1 and E2F-4 as novel HCF-1 interaction partners. These proteins most likely function in conjunction with the full complement of HCF-1 interaction partners in HCF-1-mediated cellular proliferation since this is likely a highly complex process that involves the cooperative interplay with many effector targets that recognize both the kelch and basic domains. As a result of these interactions, it is envisioned that HCF-1 will regulate the expression of a large set of target genes and influence several cellular processes including chromatin modification and pre-mRNA processing that are required for cell cycle progression. A fuller understanding of HCF-1 function in cellular proliferation will be achieved when we ascertain the function and interplay of the multitude of HCF-1 interactions in HCF-1-mediated cellular proliferation and how these interactions are regulated throughout the cell cycle by extracellular signaling pathways.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and reagents

The following is a list of chemicals and reagents used in this research, and their suppliers.

Ampicillin	Sigma
BioRad protein assay reagent	BioRad
Bovine serum albumin	Pharmacia Biotech
Calf serum	Gibco BRL
Chloramphenicol	Sigma
Complete mini (EDTA-free) protease inhibitor	Boehringer Mannheim
Coomassie brilliant blue	BDH Chemicals
Crystal violet	BDH Chemicals
ECL reagent	Amersham Life Sciences
Fetal bovine serum	Sigma
Geneticin (G418)	Gibco BRL
Glutathione-sepharose 4B	Pharmacia Biotech
Hoechst 33258	Fisher Scientific
IPTG (isopropyl-β-D-thiogalactoside)	Biosynth
Kanamycin	Boehringer Mannheim
L-glutamine	Gibco BRL
Luciferin	Biosynth
Lysozyme	Sigma
Molecular weight standards:	
1Kb$^+$ DNA ladder	Gibco BRL
BenchmarkTM protein ladder	Life Technologies
BenchmarkTM prestained protein ladder	Life Technologies
Nucleoside triphosphates	Pharmacia
ONPG (o-nitrophenyl-β-D-galactoside)	Sigma
Penicillin/streptomycin	Gibco BRL
PMSF (phenylmethylsulphonylfluoride)	Boehringer Mannheim
Polyethylene glycol (PEG)-3350	Sigma
Protein G-sepharose	Roche
Reporter lysis buffer (luciferase assay reagent)	Promega
Salmon testes DNA	Sigma
Sodium deoxycholate (DOC)	Sigma
Trypsin-EDTA	Gibco BRL
X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside)	Biosynth
3-amino-1,2,4-triazole	Sigma

2.1.2 Radiochemicals

^{35}S-methionine (1000Ci/mmol) at 10μCi/μL	Amersham Life Sciences
^{14}C-labelled protein ladder	Gibco BRL

2.1.3 Enzymes

Restriction endonucleases and modifying enzymes were purchased from New England Biolabs (NEB). Enzymes not purchased from NEB include: T7 RNA polymerase (Promega), SP6 RNA polymerase (Promega), PFU DNA polymerase (Stratagene), and RNAase A (Pharmacia Biotech).

2.1.4 Antibodies

αGAL4DBD (RKC51) (Santa Cruz Biotech) is a mouse monoclonal antibody directed against the GAL4 DNA binding domain used at a concentration of 0.1μg/mL in Western blotting. αV5 (Invitrogen) is a mouse monoclonal antibody directed against a 14 amino acid sequence (GKPIPNPLLGLDST) found in the P and V proteins of the paramyxovirus, SV5 used at a concentration of 0.1μg/mL in Western blotting. αFLAG M2 (Sigma) is a mouse monoclonal antibody raised against a FLAG octapeptide, DYKDDDDK, used at a concentration of 0.5μg/mL. αHA (12CA5) (Roche) is a mouse monoclonal antibody directed against the hemagglutinin protein of human influenza virus used at a concentration of 1μg/mL. αE2F-4 (A-20) (Santa Cruz Biotech) is a rabbit polyclonal antibody directed against the C-terminal portion of the human E2F-4 protein used at a concentration of 0.5μg/mL. Anti-mouse Ig and anti-rabbit Ig secondary antibodies from sheep and donkey respectively (Amersham Life Sciences), were horseradish peroxidase (HRP)-linked and used at a dilution of 1:10000.

2.1.5 Oligonucleotides

2.1.5.1 *Preparation of oligonucleotides*

Oligonucleotides were purchased from MOBIX (McMaster University Central Facility) purified by OPC (trityl column purification). Oligonucleotide concentrations were determined using the formula C = OD/ε, where OD refers to the optical density of the oligonucleotide as provided by the manufacturer, ε is the extinction coefficient of the oligonucleotide calculated as described (Ausubel *et al.*, 1994), and C is the concentration in mol/L. Oligonucleotides were purchased from MOBIX and resuspended in sterile water to a final concentration of 100μM.

Table 1: Oligonucleotides for site-directed mutagenesis[a]

Code #	Sequence 5'→3'	Mutation Created
AB27038	GGCACGACCACCATCGCCGCAGCCATCCCCATGTCGGCC	HCF-1 769-771A
AB27054	CACCATCATCAAAACCGCCGCCGCGTCGGCCATCATCACC	HCF-1 772-774A
AB27056	CAAAACCATCCCCATGGCGGCCGCCATCACCCAGGCGGGC	HCF-1 775-777A
AB27073	CCCATGTCGGCCATCGCCGCCGCGGCGGGCGCCACGGGTG	HCF-1 778-780A
AB27070	GCCATCATCACCCAGGCGGCCGCCACGGGTGTGACC	HCF-1 781-783A
AB27075	CACCCAGGCGGGCGCCGCGGCTGCGACCAGCAGTCCTGGC	HCF-1 784-786A
AB27204	GCGGGCGCCACGGGTGCGGCCAGCAGTCCTGGCATC	HCF-1 786-787A
AB27206	GCCACGGGTGTGACCGCCGCTCCTGGCATCAAGTCC	HCF-1 788-789A
AB26939	CGGGTGTGACCAGCAGTGCTGCCGCCAAGTCCCCCATCACCATC	HCF-1 790-792A
AB26941	CCAGCAGTCCTGGCATCGCGGCCGCCATCACCATCATCACCACC	HCF-1 793-795A
AB26820	GGCATCAAGTCCCCCGCCGCCGCCATCACCACCAAGGTG	HCF-1 796-798A
AB26931	GTCCCCCATCACCATCGCCGCCGCCAAGGTGATGACTTCAGG	HCF-1 799-801A
AB27196	GGCATCAAGTCCCCCATCAAGGTGATGACTTCAGGAAC	HCF-1 D797-801
AB27194	CATCACCATCATCACCACCGGAACTGGAGCACCTGCG	HCF-1 D802-806
AB27200	GGCATCAAGTCCCCCATCGGAACTGGAGCACCTGCG	HCF-1 D797-806
AB23873	CAAAAACGGGCCCCCTTCGTGTCCTCGACTC	HCF-1 P134S
ML077	CCGGGAGACCACGATGCTATCTACAACCTGGACG	E2F-4 Y392A

a. Nomenclature for mutations generated is as follows: For single amino acid mutations the wild type amino acid precedes the numerical position, followed by the mutated amino acid, in single letter amino acid code. For triple alanine substitutions, the residues mutated are indicated numerically, followed by the single letter amino acid code for alanine. For amino acid deletion mutants, the deleted residues are indicated numerically, preceded by Δ. Please note that complementary oligonucleotides are not shown.

Table 2: Oligonucleotides used to PCR amplify HCF-1 fragments for cloning

Code #	Sequence 5'→3'	Function
AB15229	GGATCCGAATTCCAGGCTGCCCCCGCACCCCCG	HCF-1 residue 450 (F)[a]
AB15230	GAATTCGTCGACGTTGGAAGTGACAGTGGTGGC	HCF-1 residue 1439 (R)[b]
AB13348	GAATTCGGATCCATGGCTTCGGCCGTGTCGCCCGCC	HCF-1 residue 1 (F)
AB13345	GAATTCGTCGACCTCGAGTCACAGGGAGTTGGTGTTGGCGCG	HCF-1 residue 380 (R)
AB17516	GCGAATTCGGATCCAGTGCTGTGGCCCCCCGCCCC	HCF-1 residue 902 (R)
AB17627	GCGGATCCGAATTCATCCTGGGCATCAGCAGCGTC	HCF-1 residue 750 (F)
AB17420	GGATTCGAATTCAAGGGGGCCCCGGGACAGCCA	HCF-1 residue 836 (R)
AB24712	GCGGATCCAGTGATGATTTTCGCAGGTGC	HCF-1 residue 816 (R)
AB25909	GCGGATCCAGGTGCTCCAGTTCCTGAAGT	HCF-1 residue 811 (R)
AB25910	GCGGATCCTGAAGTCATCACCTTGGTGGT	HCF-1 residue 806 (R)
AB25911	GCGGTGGTGATGATGGTGATGG	HCF-1 residue 801 (R)
AB24713	GCGGATCCGATGGGGGACTTGATGCCAGG	HCF-1 residue 796 (R)
AB24709	GCGAATTCAAAACCATCCCCATGTCGGCC	HCF-1 residue 770 (F)
AB24710	GCGAATTCCCCTGGCATCAAGTCCCCCATC	HCF-1 residue 790 (F)
AB17421	GAATTCGGATCCAGTGCTGTGGCCCCCCGCCCC	HCF-1 residue 902 (R)
AB19883	GCGGGATCCCTCGAGCTTAAGCACCACCTGGGTCAC	HCF-1 residue 836 (R)
AB17472	GCGGATCCGAATTCCAGGCTGCCCCCGCACCCCCG	HCF-1 residue 450 (F)
AB17473	GCGAATTCCTCGAGAAGCACCACCTGGGTCACTCC	HCF-1 residue 836 (R)
AB24438	GCGAATTCGATATCTCGCTTCGGCCGTGTCGCCCGCC	HCF-1 residue 2 (F)
AB24439	GCGGATCCGATATCAGTGCTGTGGCCCCCCGCCCC	HCF-1 residue 902 (R)
AB23963	GCGGATCCCTCGAGAGTGCTGTGGCCCCCCGC	HCF-1 residue 902 (R)
AB23998	GCGAATTCCTCGAGATCCTGGGCATCAGCAGCGTC	HCF-1 residue 750 (F)

a. (F) indicates the primer functions as a forward primer in PCR reactions and starts with the residue indicated.
b. (R) indicates the primer functions as a reverse primer in PCR reactions and ends with the residue indicated.

Table 3: Oligonucleotides used to PCR amplify HCF-2 fragments for cloning

Code #	Sequence 5'→3'	Function
AB30435	GCGGATCCCTCGAGGGAGTTGGTAGTGGCTTTG	HCF-2 residue 373 (R)[a]
AB25638	GCGGATCCTCAATTTAAAGGTGCTTTCTTATTG	HCF-2 residue 792 (R)
AB30434	GCGAATTCCTCGAGTTTCATGTCAAGTGGGAT	HCF-2 residue 373 (F)[b]
AB30138	GCTCCCGCTTTCACTGTGGCCAC	HCF-2 residue 596 (R)
AB30140	GCGAATTCATAGGTCCTTTCAGCAAAATC	HCF-2 residue 662 (F)
AB30141	GCGAATTCAGTCAACTTGTGTTCATGAGG	HCF-2 residue 727 (F)
AB28948	GCGAATTCGAACGACAATGGTGTGATGTG	HCF-2 residue 596 (F)
AB30349	GCGAATTCCTCGAGGCGGCTCCCAGCCTCCTCAAC	HCF-2 residue 2 (F)
ML894	GCGAATTCGCATGCTGGCGGCTCCCAGCCTCCTCAAC	HCF-2 residue 2 (F)
ML895	GCGGATCCTCAGCAGTTGGTAGTGGCTTT	HCF-2 residue 373 (R)

a. (R) indicates the primer functions as a reverse primer in PCR reactions and ends with the residue indicated.
b. (F) indicates the primer functions as a forward primer in PCR reactions and starts with the residue indicated.

Table 4: Oligonucleotides used to PCR amplify Miz-1 fragments for cloning

Code #	Sequence 5'→3'	Function
AB17506	GCGGATCCCTCGACACCATGGACTTTCCCCAGCACAGC	Miz-1 residue 1 (F)[a]
AB20155	GCGGGATCCGCCATGGCCCATGCCCTCAAGTCACTTGCT	Miz-1 residue 109 (F)
AB18629	GGGAATTCGGATCCCATGCCCTCAAGTCACTTGCT	Miz-1 residue 109 (F)
AB20884	GCGGGATCCCTTGTGGATGACGGAGCCGTA	Miz-1 residue 308 (R)[b]
AB21451	GCGAATTCGCCATGGCCGCGGCCAGCGGTGCAGAGCAG	Miz-1 residue 176 (F)
AB21452	GCGGATCCGGCCTCGGAGCCGAGCTCCTG	Miz-1 residue 284 (R)
AB20156	GCGGGATCCGAATTCCTTGTGGATGACGGAGCCGTA	Miz-1 residue 308 (R)
AB17507	GCGAATTCAGATCTTCACTCGGCAGGCGGGGGACA	Miz-1 residue 803 (R)
AB22595	GCGAATTCGCCATGGCGTGTGACTCCTGTGGGGACAAG	Miz-1 residue 719 (F)
AB22596	GCGGATCCGTGGATTCGCACATGCTGAGC	Miz-1 residue 738 (R)
AB22536	GCGAATTCGCCATGGCGAAAGCTGTGAAGCAAGTGCAG	Miz-1 residue 701 (F)
AB21813	GCGAATTCGCCATGGACTTTCCCCAGCACAGC	Miz-1 residue 1 (F)
AB21814	GCGGATCCGCGGCCGCGCAGGCCGTGATGATGTCCTG	Miz-1 residue 109 (R)
AB21815	GCGAATTCGCCATGGCGCAGGGCAAGGCAGGCATCAAG	Miz-1 residue 637 (F)
AB21816	GCGGATCCTCACTCGGCAGGCGGGGGACA	Miz-1 residue 803 (R)
AB21817	GCGGATCCTCAGGCGTAGAGGATGTGAGTGTT	Miz-1 residue 718 (R)

a. (F) indicates the primer functions as a forward primer in PCR reactions and starts with the residue indicated.
b. (R) indicates the primer functions as a reverse primer in PCR reactions and ends with the residue indicated.

Table 5: Sequencing Primers

Code #	Sequence 5'→3'	Function
ML434	CCCCGCCCCGTTGACGC	5' primer for pFLAG-CMV-6c
AB18034	TCACCGAAACGCGCGAGG	3' primer for pGEX
AB9324	GGGCTGGCAAGCCACGTTGGTG	5' primer for pGEX
AB14974	GAAGAGAGTAGTAACAAAGG	5' primer for pGBT9
AB16219	TTCGCCCGGAATTAGCTT	3' primer for pGBT9
AB18969	GATGTATATAACTATCTA	5' primer for pGADGH
M13 forward primer	GTAAAACGACGGCCAGT	3'primer for pGADGH

2.1.6 Plasmids

2.1.6.1 *Vectors*

pGBT9 (Clontech): A yeast vector used in the yeast 2-hybrid system to express GAL4 DNA binding domain (amino acids 1-147) fusions. Contains a multiple cloning

site (MCS) downstream of the GAL4 DBD coding region and a tryptophan marker for selection on auxotrophic media.

pGEX (Pharmacia Biotech): A bacterial vector that permits inducible protein expression with IPTG, used to express GST fusions. An MCS is present downstream of the GST coding region.

pGEM5zf (Promega): A vector used for *in vitro* transcription/translation reactions containing a T7 RNA polymerase promoter upstream from an MCS.

pSPUTK: A vector used for *in vitro* transcription/translation reactions containing an SP6 RNA polymerase promoter upstream from an MCS, kindly provided by Dr. David Andrews (McMaster University).

pSG424: A mammalian expression vector used to express GAL4 DNA binding domain (amino acids 1-147) fusions as described (Sadowski and Ptashne, 1989), provided by Dr. John Hassell (McMaster University). Contains a multiple cloning site (MCS) downstream of the GAL4 DBD coding region.

pFLAG-CMV-6c (Sigma): A mammalian expression vector used to express the protein of interest with a N-terminal FLAG tag. Contains a multiple cloning site (MCS) downstream of the FLAG coding region.

pCMV-myc-Nuc (Invitrogen): A mammalian expression vector used to express the protein of interest with a C-terminal myc tag and tandem nuclear localization sequences. Contains a multiple cloning site (MCS) upstream of the myc and NLS coding regions.

2.1.6.2 *Plasmids from other sources*

pSV2-NEO (Clontech): A vector containing the neomycin resistance cassette used to select transfected mammalian cells under selection with geneticin (G418).

pGAD424-VP16$_{1-404}$: A yeast expression vector for an N-terminal fragment of VP16 spanning residues 1-404 fused to the GAL4 activation domain was provided by J. Schmelter (Schmelter *et al.*, 1996).

pTOPO-Miz-1$_{FL \text{ and } \Delta POZ}$: Mammalian expression vectors for full length Miz-1 and Miz-1 ΔPOZ were provided by Dr. Robert Tjian (University of California, Berkeley) (Ziegelbauer *et al.*, 2001).

pFLAG-C1: A mammalian expression vector for full length HCF-1 (C1) (spanning residues 3-2035) containing an N-terminal FLAG tag was provided by Dr. Tom Kristie (NIH, Maryland).

pGADGH-GABP(β2)$_{60-347}$: A yeast expression vector for GABP(β2) spanning residues 60-347 isolated from a yeast 2-hybrid screen using HCF-1 as bait was provided by Dr. Tom Kristie (Vogel and Kristie, 2000a).

p(5x)GAL4LUC: A luciferase reporter gene plasmid containing five repeated GAL4 response elements upstream of the luciferase coding region, provided by Dr. John Hassell.

pSG424-VP16$_{AAD}$: A mammalian expression vector for a GAL4 DBD fusion to the VP16 acidic activation domain, provided by Dr. I. Sadowski, UBC (Sadowski and Ptashne, 1989).

$p15^{INK4b}$LUC: A luciferase reporter gene plasmid containing the human $p15^{INK4b}$ promoter spanning -113/+60 upstream of the luciferase coding region (Staller *et al.*, 2001) provided by Dr. Martin Eilers (Institute of Molecular Biology and Tumour Research, Marburg, Germany).

pPK7-Miz-1: A mammalian expression vector for full length Miz-1 (Peukert *et al.*, 1997) provided by Dr. Martin Eilers.

pMNBabeIRESc-myc: A mammalian expression vector for full length c-myc provided by Dr. Linda Penn (University of Toronto).

p300-VP16: A mammalian expression vector for p300 fused to VP16 for use in the mammalian 2-hybrid assay for interaction with GAL4DBD-Miz-1, provided by Dr. Robert Eckner (University of Zurich).

pGEX-$p300_{1572-2371}$: A bacterial expression vector for a fragment of p300 spanning residues 1572-2371 fused to GST was provided by Dr. Robert Eckner.

pcDNA-myc: A plasmid used in *in vitro* transcription/translation reactions with T7 RNA polymerase to produce full length myc was provided by Dr. Martin Eilers.

pGEX-$VP16_{1-404}$: A bacterial expression vector for an N-terminal fragment of VP16 spanning residues 1-404 fused to GST was provided by J. Knez (Knez *et al.*, 2003).

pSPUTK-HCF-$1_{612-902}$: A fragment encompassing HCF-1 residues 612-902 was isolated by digesting pGBT9-HCF-$1_{450-902}$ plasmid with *PstI*/*Bam*HI (site located in the

3' primer) and inserted into pSPUTK digested with *PstI*/*Bam*HI. This construct was created by Sushmita Pamidi.

pCGN-HCF-1: A mammalian expression vector for full length HCF-1 containing an N-terminal HA tag and a C-terminal myc tag (Wilson *et al.*, 1997) was provided by Dr. Winship Herr (Cold Spring Harbor Laboratory).

pCGN-HCF-$1_{1\text{-}1011}$: A mammalian expression vector for an N-terminal fragment of HCF-1 spanning residues 1-1011 containing an N-terminal HA tag (Wilson *et al.*, 1997) was provided by Dr. Angus Wilson (New York University).

pCGN-HCF-$1_{1\text{-}1011P134S}$: Identical to the plasmid described above except the HCF-1 coding sequence contains the P134S mutation in which proline-134 is changed to serine (Johnson *et al.*, 1999). This plasmid was also provided by Dr. Angus Wilson.

pCGN-HCF-1_{P134S}: This mammalian expression vector is identical to pCGN-HCF-1 except the HCF-1 coding sequence contains the P134S mutation in which proline-134 is changed to serine. It was constructed by Jozo Knez by isolating a *SpeI*/*Xho*I fragment from pCGN-HCF-1 and inserting this fragment into pCGN-HCF-$1_{1\text{-}1011P134S}$.

pCGN-HCF-2: A mammalian expression vector for full length HCF-2 containing an N-terminal HA tag (Johnson *et al.*, 1999) was provided by Dr. Angus Wilson.

pCMV-HA-E2F-1; pCMV-HA-E2F-4; pCMV-HA-E2F-5: Mammalian expression vectors for full length E2F protein containing N-terminal HA tags provided by Dr. Peter Whyte (McMaster University).

pGEX-E2F-4_{C70}: A bacterial expression vector producing a GST fusion to the C-terminal 70 amino acids of E2F-4, provided by Dr. Peter Whyte (McMaster University).

pBSK-p130: A plasmid used in *in vitro* transcription/translation reactions with T7 RNA polymerase to produce full length p130 was provided by Dr. Peter Whyte (McMaster University).

2.1.6.3 *Plasmids isolated by yeast 2-hybrid screening*

pGADGH-Miz-$1_{269-803}$: A plasmid isolated in the HCF-$1_{450-1439}$ screen which encodes amino acids 269-803 of Miz-1 fused to the GAL4 activation domain and contains a leucine marker.

pGADGH-ZF_{9-272}: A plasmid isolated in the HCF-1 kelch domain screen which encodes amino acids 9-272 of Zhangfei (ZF) fused to the GAL4 activation domain and contains a leucine marker.

2.1.6.4 *Plasmids constructed for research*

2.1.6.4.1 *Yeast 2-hybrid plasmids*

pGBT9-HCF-1 (450-1439): An HCF-1 fragment encoding amino acids 450-1439 was produced by PCR with pCGN-HCF-1 as template using AB15229/AB15230 primers, digested with *Eco*RI/*Sal*I, and inserted into pGBT9.

pGBT9-HCF-1(1-380): An HCF-1 fragment encoding amino acids 1-380 was excised from pGEM5zf-HCF-1_{1-380} using *Eco*RI/*Sal*I and inserted into pGBT9.

pGBT9-HCF-1 (1-902): HCF-1 fragment 1-902 was produced by PCR with pCGN-HCF-1 as template using AB23800/AB17516 primers, digested with *Eco*RI/*Bam*HI, and inserted into pGBT9.

pGBT9-HCF-1 (450-902; 750-902; 450-836; 750-836; 836-902; 750-816; 750-811; 750-806; 750-801; 750-796; 770-836; 790-836; and 790-902): HCF-1 fragments were produced by PCR with pCGN-HCF-1 as template using AB15229/AB17516, AB17627/AB17516, AB17472/AB17473, AB17627/AB19883, AB17420/AB17421, AB17627/AB24712, AB17627/AB25909, AB17627/AB25910, AB17627/AB25911, AB17627/AB24713, AB24709/AB19883, AB24710/AB19883, and AB24710/AB17516 primers respectively, digested with *Eco*RI/*Bam*HI, and inserted into pGBT9 to generate pGBT9 HCF-1 derivatives.

pGBT9-HCF-2 (2-373; 373-596; 596-792; 662-792; and 727-792): HCF-2 fragments were produced by PCR with pCGN-HCF-2 as template using AB30349/30435, AB30434/AB30138, AB28948/AB25638, AB30140/AB25638, and AB30141/AB25638 primers respectively, digested with *Eco*RI/*Bam*HI, and inserted into pGBT9 to generate pGBT9 HCF-2 derivatives.

pGBT9-HCF-$1_{750\text{-}902}$ (769-771Ala; 772-774Ala; 775-777Ala; 778-780Ala: 781-783Ala; 784-786Ala; 786-787Ala; 788-789Ala; 790-792Ala; 793-795Ala; 796-798Ala; 799-801Ala; Δ797-801; Δ802-806; Δ797-806): Mutants 769-771Ala, 772-774Ala, 775-777Ala, 778-780Ala, 781-783Ala, 784-786Ala, 786-787Ala, 788-789Ala, 790-792Ala, 793-795Ala, 796-798Ala, 799-801Ala, Δ797-801, and Δ802-806 within the GAL4DBD-

HCF-$1_{750\text{-}902}$ polypeptide were constructed using the Stratagene QuikChange Method as described by the manufacturer with pGBT9-HCF-$1_{750\text{-}902}$ as template using AB27038, AB27054, AB27056, AB27073, AB27070, AB27075, AB27204, AB27206, AB26939, AB26941, AB26820, AB26931, AB27196, and AB27194 as primers (along with the complementary primer) respectively. Mutant Δ797-806 was constructed with pGBT9-HCF-$1_{750\text{-}902}$(Δ797-801) as template and AB27200 as primer (along with the complementary primer) to delete residues 802-806.

pGBT9-Miz-1_{FL}: A fragment encompassing Miz-1 full length was isolated from pPK7-Miz-1 by digestion with *Eco*RI/*Bam*HI (a partial digest was done for *Bam*HI in which the plasmid was digested with a 1/10 dilution of *Bam*HI for only 10 minutes since the Miz-1 fragment contained an internal *Bam*HI site in addition to the *Bam*HI site at the 3' end), and inserted into pGBT9.

pGBT9-Miz-$1_{\Delta POZ}$: A fragment encoding Miz-1 residues 110-803 was isolated from pEGFP-C2-Miz-$1_{\Delta POZ}$ by digestion with *Eco*RI and inserted into pGBT9.

pGBT9-Miz-1 (1-109; 176-284; 637-803; 637-718; 637-738; 701-803; and 701-738; 719-803): Miz-1 fragments were produced by PCR with pPK7-Miz-1 as template using AB21813/AB21814, AB21451/AB21452, AB21815/AB21819, AB81815/AB21817, AB21815/AB22596, AB22536/21816, AB22536/AB22596 and AB22595/AB21816 primers respectively, digested with *Eco*RI/*Bam*HI, and inserted into pGBT9 to generate pGBT9-Miz-1 derivatives.

2.1.6.4.2 *GST pull-down assay plasmids*

pGEX4T1-HCF-1 (750-902 and 770-836): Fragments of HCF-1 were excised from the corresponding pGBT9-HCF-1 clones with *Eco*RI/*Sal*I and inserted into pGEX4T1 to generate pGEX4T1-HCF-1 derivatives.

pGEX4T1-Miz-1_{POZ}: A fragment of Miz-1 encoding residues 1-109 (POZ) was excised from pGBT9-Miz-1_{POZ} with *Eco*RI/*Not*I and inserted into pGEX4T1.

pGEX4T1-Miz-1 (637-803; 637-738; and 719-803): Fragments of Miz-1 encoding residues 637-803, 637-738, and 719-803 were excised from the corresponding pGBT9-Miz-1 plasmids with *Eco*RI/*Sal*I and inserted into pGEX4T1.

pGEX4T1-HCF-2 (373-596; 596-792; 727-792; and 662-792): Fragments of HCF-2 encoding residues 373-596, 596-792, 727-792, and 662-792 were excised from the corresponding pGBT9-HCF-2 plasmids with *Eco*RI/*Sal*I and inserted into pGEX4T1.

pSPUTK-HCF-2 (FL and 2-373): HCF-2 full length and 2-373 fragments were produced by PCR with pCGN-HCF-2 as template using ML894/AB25638 and ML894/ML895 respectively as primers, digested with *Sph*I/*Bam*HI and inserted into pSPUTK digested with *Sph*I/*Bam*HI.

pGEM5zf-Miz-1_{FL}: pGADGH-Miz-$1_{269-803}$ was digested with *Not*I/*Xho*I to isolate the C-terminal fragment of Miz-1, and pPK7-Miz-1 was digested with *Nco*1/*Not*I to isolate the N-terminal fragment. These two fragments were ligated to each other using the common *Not*I site to generate a fragment of full length Miz-1 which was inserted into

pGEM5zf digested with *NcoI/SalI* (which gives the same cohesive end as the *XhoI* digest) to yield the full length Miz-1 coding sequence downstream of a T7 promoter for use in *in vitro* transcription/translation reactions.

pSPUTK-Miz-1 (POZ and 637-803): Fragments encoding the Miz-1 POZ domain (residues 1-109) and residues 637-803 were isolated by digesting the corresponding pGBT9-Miz-1 plasmids with *NcoI/Bam*HI and inserted into pSPUTK.

pSPUTK-Miz-$1_{109\text{-}308}$: A fragment encoding Miz-1 residues 109-308 was generated by PCR with pPK7-Miz-1 as template using primers AB20155/AB20156, digested with *Bam*HI/*Eco*RI, and inserted temporarily into pCANHA1 (a vector supplied by Dr. J. Hassell). The fragment was subcloned from pCANHA1 into pSPUTK digested with *NcoI/Eco*RI, by isolating the fragment by digestion with *NcoI/Eco*RI.

pGEM5zf-HCF-$1_{1\text{-}902}$: A fragment of HCF-1 encoding residues 1-902 was generated by PCR with pCGN-HCF-1 as template using AB24438/AB24439 as primers, digested with *Eco*RV, and inserted into pGEM5zf.

pGEM5zf-HCF-$1_{1\text{-}902P134S}$: A P134S mutation was generated in HCF-$1_{1\text{-}902}$ using the Stratagene QuikChange Method as described by the manufacturer with pGEM5zf-HCF-$1_{1\text{-}902}$ as template using AB23873 as primer (and its complementary primer).

pGEM5zf-HCF-$1_{1\text{-}380}$: A fragment of HCF-1 encoding residues 1-380 was generated by PCR with pCGN-HCF-1 as template using AB13348/AB13345 as primers, phosphorylated and ligated into pGEM5zf digested with *Eco*RV.

pGEX-E2F-$4_{C70Y392A}$: A Y392A mutation was generated in E2F-4_{C70} using the Stratagene QuikChange Method as described by the manufacturer with pGEX-E2F-4_{C70} as template using ML077 as primer (and its complementary primer).

2.1.6.4.3 *Mammalian expression plasmids*

pSG424-Miz-1_{FL}: A fragment of Miz-1 full length was excised from the corresponding pGBT9-Miz-1 plasmid with *Xho*I (which is located in the GAL4 DBD) and *Bam*HI (a partial digest was done for *Bam*HI in which the plasmid was digested with a 1/10 dilution of *Bam*HI for only 10 minutes since the Miz-1 fragment contained an internal *Bam*HI site in addition to the *Bam*HI site at the 3' end), and inserted into pSG424.

pSG424-Miz-$1_{\Delta POZ}$: A fragment of Miz-1 encoding residues 110-803 was excised from the corresponding pGBT9-Miz-1 plasmid with *Xho*I (which is located in the GAL4 DBD) and *Bam*HI (a partial digest was done for *Bam*HI in which the plasmid was digested with a 1/10 dilution of *Bam*HI for only 10 minutes since the Miz-1 fragment contained an internal *Bam*HI site in addition to the *Bam*HI site at the 3' end), and inserted into pSG424.

pSG424-Miz-1 (176-284; 637-803; 637-718; 701-803; and 701-738): Fragments of Miz-1 encoding residues 176-284, 637-803, 637-718, 701-803, and 701-738 were excised from the corresponding pGBT9-Miz-1 plasmids with *Xho*I (which is located in the GAL4 DBD)/*Bam*HI and inserted into pSG424.

pCMV/myc/nuc-HCF-$1_{750\text{-}902}$: A fragment of HCF-1 encoding residue 750-902 was generated by PCR with pCGN-HCF-1 as template using AB23998/AB23963 as primers, digested with *Xho*I and inserted into pCMV/myc/nuc plasmid.

pFLAG-CMV-6c-HCF-1 (1-902; 450-902; 750-902 and 770-836): Fragments of HCF-1 encoding residues 1-902, 450-902, 750-902, and 770-836 were excised from the corresponding pGBT9-HCF-1 plasmids with *Eco*RI/*Bam*HI and inserted into pFLAG-CMV-6c.

pFLAG-CMV-6c-HCF-$1_{1\text{-}902P134S}$: A P134S mutation was generated in the pFLAG-CMV-6c-HCF-$1_{1\text{-}902}$ using the Stratagene QuikChange Method as described by the manufacturer using AB23873 as primer (and its complementary primer).

pFLAG-CMV-6c-HCF-$1_{1\text{-}380}$: A fragment of HCF-1 encoding residues 1-380 was excised from the corresponding pGBT9-HCF-1 plasmid with *Eco*RI/*Sal*I and inserted into pFLAG-CMV-6c.

pFLAG-CMV-6c-HCF-$1_{1\text{-}380P134S}$: A P134S mutation was generated in the pFLAG-CMV-6c-HCF-$1_{1\text{-}380}$ using the Stratagene QuikChange Method as described by the manufacturer using AB23873 as primer (and its complementary primer).

pFLAG-CMV-6c-HCF-2 (2-373; 373-596; 662-792): Fragments of HCF-2 encoding residues 2-373, 373-596, and 662-792 were excised from the corresponding pGBT9-HCF-2 plasmids with *Eco*RI/*Bam*HI and inserted into pFLAG-CMV-6c.

pCGN-HCF-$1_{1\text{-}1011}$ (769-771Ala; 781-783Ala; and 793-795Ala): Mutants in pCGN-HCF-$1_{1\text{-}1011}$ were generated using the Stratagene QuikChange Method as described by the manufacturer using AB27038; AB27070; and AB26941 (along with the complementary primers) respectively as primers.

pCMV-HA-E2F-4 (Y392A): A Y392A mutation was generated in pCMV-HA-E2F-4 by PCR mutagenesis using ML077 (along with its complementary primer).

pEGFP-C2-Miz-$1_{\Delta POZ}$: A Miz-$1_{\Delta POZ}$ fragment was generated by PCR with pPK7-Miz-1 as template using AB18629/AB17507, digested with *Eco*RI and inserted into pEGFP-C2. This plasmid contains a kanamycin resistance cassette.

2.1.7 Organisms and Strains

2.1.7.1 *Bacteria*

Escherichia coli (*E. coli*) **DH5α** was used in plasmid cloning and amplification unless otherwise noted. Strain **HB101** is *leuB* deficient and was therefore used to select pGADGH plasmids isolated from yeast containing both pGBT9 and pGADGH plasmids. Strain **BL21(DE3)** (Novagen) was specifically used to express GST fusion proteins since

it lacks *lon* and *ompT* proteases and allows for IPTG-inducible expression of GST fusion proteins from pGEX plasmids.

2.1.7.2 *Yeast*

Saccharomyces cerevisiae **Y190** (*MATα, leu2-3,112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δ80Δ URA3 GAL-lacZ, LYS GAL-HIS3, CYH^r*) and **HF7c** (*MATα, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112, gal4-52, gal80-538, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3::GAL4$_{17mers(x3)}$-CyC1$_{TATA}$-lacZ*) were used in yeast 2-hybrid studies. Both strains contain β-galactosidase and His3 reporter genes under the control of upstream activation sequences for GAL4 (UAS_G). HF7c was used for the yeast 2-hybrid screen for HCF-1-interacting proteins due to its more stringent control of His3 expression compared to Y190 (Feilotter *et al.*, 1994). However, Y190 was used in protein-protein interaction studies due to its easily detectable levels of β-galactosidase activity.

2.1.7.3 *Mammalian cells*

Cos-1 cells are CV-1 derived African green monkey (*Cercopithecus aethiops*) kidney cells that have been transformed with SV40 (Gluzman, 1981). These cells were obtained from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine.

tsBN67$_{HR1}$ cells are a temperature-sensitive cell line derived from BHK21(baby hamster kidney fibroblast) cells. tsBN67$_{HR1}$ cells arrest cell cycle progression at G0/G1 at the non-permissive temperature (40°C) due to a missense mutation in the only copy of the *HCF-1* gene in which proline 134 is mutated to serine (Goto *et al.*, 1997). tsBN67$_{HR1}$ are a clonal isolate of tsBN67 cells which, at the time of their isolation, did not contain any revertant colonies (Reilly and Herr, 2002). These cells were supplied by Dr. Winship Herr and grown in DMEM supplemented with 10% calf serum (CS), 1% penicillin-streptomycin, and 1% L-glutamine at the permissive temperature (33.5°C).

2.2 METHODS

2.2.1 Maintenance and growth of bacterial cells

DH5α and BL21(DE3) were grown at 37°C using 2YT media as described (Sambrook *et al.*, 1989). HB101 were grown at 37°C using M9 minimal media supplemented with 1mM thiamine-HCl, 40μg/mL proline, and 0.4% glucose as described (Sambrook *et al.*, 1989). Plasmids were maintained within bacteria with 100μg/mL ampicillin.

2.2.2 Plasmid DNA Purification

2.2.2.1 *Small scale plasmid preparation*

Minipreps were performed using the lysis boiling method as described (Sambrook *et al.*, 1989). Briefly, a single colony of bacteria containing the plasmid of interest was used to inoculate 5mL 2YT AMP, and grown overnight at 37°C with moderate agitation. 1.5mL of the overnight culture was transferred to a microcentrifuge tube and harvested by centrifugation at high speed. Pellets were resuspended in 350μL of lysis buffer

(10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 100mM NaCl, and 5% Triton X-100) and 30μL of 10mg/mL lysozyme (prepared fresh in 10mM Tris-HCl pH 8.0) was added. The cell suspension was incubated in a boiling water bath for 40 seconds, harvested as before, and the pellet of bacterial debris was removed with a sterile toothpick. Plasmid DNA, which remained in the supernatant, was precipitated with 200μL of 7.5M ammonium acetate and 600μL of isopropanol, and flash frozen in liquid nitrogen. To recover the nucleic acids, the mixture was centrifuged at high speed for 30 minutes at 4°C. The resulting pellet was rinsed with 1mL of 70% ethanol, and resuspended in TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, and 50μg/mL RNAase A).

2.2.2.2 *Large scale plasmid preparation*

Large scale plasmid preparations were performed using a commercially available kit (Qiagen), based on the alkaline lysis method and anion exchange chromatography (Birnboim, 1983). Briefly, a 500mL overnight bacterial culture was harvested by centrifugation at 3000 x g at 4°C for 5 minutes and subjected to lysis. Subsequently, the contents were centrifuged at high speed to pellet cellular debris. The supernatant, containing plasmid DNA, was added to an anion exchange column supplied by the manufacturer (Qiagen) and plasmid DNA eluted as instructed by the manufacturer. The resulting eluate was combined with 0.7 volume of isopropanol, centrifuged, and the pellet was rinsed with 70% ethanol and left to dry. The purified plasmid DNA was resuspended in sterile TE (pH 8.0) and quantified by fluorometry.

2.2.2.3 *Phenol/chloroform DNA extraction*

Phenol/chloroform extractions of nucleic acids were performed as previously described (Sambrook *et al.*, 1989). Crude yeast DNA samples were brought to a volume of 400µL with sterile water. A 1:1 mixture of 400µL of phenol/chloroform was added to the sample, the tube was vortexed, and then centrifuged at high speed. The upper aqueous layer was retained and mixed with 400µL of chloroform. This mixture was vortexed, centrifuged as before, and the upper aqueous layer retained. In order to precipitate out plasmid DNA, a final concentration of 0.3M sodium acetate and 2 volumes of 95% ethanol were added to the extract, which was flash frozen in liquid nitrogen, and centrifuged at high speed. The resulting pellet was rinsed with 95% ethanol and resuspended in sterile water.

2.2.3 Chloramphenicol amplification of plasmid DNA

Chloramphenicol was added to *E. coli* cultures in order to amplify plasmid DNA and increase yields obtained from large scale plasmid DNA preparations of pSG424-based plasmids, since pSG424 is a low copy number plasmid and in the absence of following this procedure, yields of its DNA were very low. A single *E. coli* colony containing a pSG424-based plasmid was used to inoculate a 50mL culture of 2YTAMP, and grown overnight at 37°C with moderate agitation. The following day the 50mL overnight culture was added to 500mL of fresh 2YTAMP media and grown to an OD_{600} of 0.5, at which point chloramphenicol (dissolved in ethanol) was added to a final concentration of 170µg/mL. The culture was subsequently grown overnight and

harvested the following day and the pGS424-based plasmid isolated by the large scale plasmid DNA preparation protocol described earlier.

2.2.4 Fluorometry

Plasmid concentrations were quantified using a Hoefer Mini-Fluorometre (TKO 100) according to the manufacturer's instructions. Briefly, purified plasmid DNA was added to 2mL of TNE buffer (10mM Tris-HCl pH 7.4, 1mM EDTA, 100mM NaCl) containing 1μg/mL Hoechst 33258 dye. The resulting fluorescence was standardized with calf thymus DNA and used to calculate the concentration of the plasmid DNA.

2.2.5 Cloning techniques

The plasmid constructs described in the materials section were constructed using standard recombinant DNA techniques as previously described (Sambrook *et al.*, 1989).

2.2.5.1 *PCR (Polymerase Chain Reaction)*

PCR reactions were performed as previously outlined (Ausubel *et al.*, 1994). Reactions were performed with 200ng of plasmid DNA, 1μM of primers, 4mM dNTPs, between 2-6mM $MgSO_4$, and 1 unit of Vent DNA polymerase with 1X thermopol buffer. A "hot start" was initiated at 95°C for 5 minutes, followed by the addition of Vent DNA polymerase. Subsequent melting cycles were programmed for 1 minute at 95°C. The melting temperature was calculated using the equation: Tm = 81.5 + 16.6(log0.0334) + 41 (#GCs/total base pairs) – 500/total base pairs. The annealing temperature was set to 1°C below the lowest primer melting temperature. Annealing was programmed for 1 minute. The elongation cycle proceeded at 72°C for a variable time, depending on the length of the fragment to be produced (~1Kb/min), and the reaction was set for 30 cycles.

2.2.5.2 *Site-directed mutagenesis*

Site-directed mutagenesis was performed using the QuikChange PCR mutagenesis kit as suggested by the manufacturer (Stratagene). Each mutant was generated from two complementary oligonucleotides designed to possess the desired nucleotide mutations. Reactions contained 10ng of parental plasmid DNA, 200mM dNTPs, 125ng of each primer, 1X PFU enzyme buffer, 2.5 Units PFU DNA polymerase, and sterile water to a total of 50μL. A parallel reaction was also set up lacking PFU enzyme in order to determine the amount of background transformants. A hot start melting cycle was initiated at 95°C for 5 minutes after which the PFU enzyme was added to the reaction. Subsequent melting cycles were programmed for 30 seconds at 95°C. Annealing was programmed for 1 minute at 55°C, and elongation was conducted at 68°C for 2 minutes/Kb of plasmid with an additional 2-3 minutes. The number of cycles was variable, depending on the extent of mutagenesis to be performed. Thus, 15 cycles were used for single nucleotide changes, 18 for single amino acid changes, and 21 cycles for multiple amino acid changes or deletions. Following the reaction, 1μL of *Dpn*I enzyme was added to the reaction and incubated at 37°C for 3 hours to digest parental plasmid. 5μL of the reaction was then used to transform 100μL of competent DH5α bacteria, and colonies which formed were selected for DNA sequencing to isolate mutants.

2.2.5.3 *DNA extraction from agarose gels (Qiaex)*

DNA fragments were recovered from agarose gels using the Qiaex agarose gel extraction method as instructed by the manufacturer (Qiagen). Briefly, DNA fragments to be used in cloning were excised from agarose gels with a sharp scalpel and transferred

to a microcentrifuge tube followed by the addition of 300μL of buffer QX1 and 10μL of Qiaex II beads, and incubated at 50°C. The sample was harvested by centrifugation at high speed for 30 seconds and the supernatant was removed. The pellet was washed with 500μL of QX1, followed by two washes with 500μL of buffer PE. The pellet was air dried and the DNA fragments were eluted by resuspension in 20μL of 10mM Tris-HCl, pH 8.5. Finally, the sample was centrifuged for 30 seconds at high speed and the supernatant containing the DNA fragment was placed into a clean microcentrifuge tube.

2.2.6 DNA sequencing

DNA sequencing was performed by the McMaster central facility at MOBIX.

2.2.7 Introduction of Plasmid DNA into *E. Coli*

2.2.7.1 *Preparation of chemically competent cells*

A single colony of bacteria, used to inoculate 5mL of 2YT media, was grown overnight at 37°C with moderate agitation. The following day, 2mL of the overnight culture was transferred to a 2L flask containing 50mL of 2YT and incubated to an OD_{550} ~ 0.3. The culture was collected in four 50mL sterile centrifuge tubes and chilled on ice for 15 minutes. The cells were pelleted by centrifugation at 3000 x g for 5 minutes at 4°C and each pellet was resuspended in 16mL of transformation buffer 1 (100mM RbCl, 50mM $MnCl_2 \cdot 4H_20$, 10mM $CaCl_2 \cdot 2H_2O$, 30mM KAc pH 7.5, 15% glycerol, pH adjusted to 5.8 with 0.2M acetic acid), and incubated on ice for 15 minutes. The cells were harvested as above and each pellet was resuspended in 4mL of transformation buffer 2 (10mM MOPS at pH 6.8 adjusted with NaOH, 10mM RbCl, 75mM $CaCl_2 \cdot H_2O$, 15%

glycerol). The cell suspension was aliquoted into pre-chilled microcentrifuge tubes, frozen on dry ice and stored at -70°C.

2.2.7.2 ***Bacterial Transformation (Chemical)***

100μL of chemically competent bacteria were mixed with 1-5μL of plasmid DNA and incubated on ice for forty minutes. Cells were then heat shocked at 37°C for 40 seconds and returned to ice for 5 minutes, followed by the addition of 400μL of 2YT. Cells were incubated for 1 hour at 37°C with moderate agitation and 100μL of the mixture was added to 2YT AMP plates and incubated overnight at 37°C.

2.2.7.3 ***Preparation of electrocompetent cells***

A single colony of *E. coli* HB101, used to inoculate 5mL 2YT, was grown overnight at 37°C with moderate agitation. The following day, 2mL of the overnight culture was transferred to a sterile 2L flask containing 50mL of 2YT and incubated to an OD_{600} ~ 0.5-0.7. The cells were chilled on ice for 10 minutes, transferred to a pre-chilled 1L centrifuge bottle, and harvested by centrifugation at 3000 x g for 10 minutes. The cells were resuspended in 500mL ice cold water twice and pelleted as before. Next, the cells were resuspended in 40mL ice cold 10% glycerol and pelleted as before. Finally, the pellet was resuspended in an equal volume of ice cold 10% glycerol and cells aliquoted into pre-chilled microcentrifuge tubes, frozen on dry ice, and stored at -70°C.

2.2.7.4 ***Bacterial transformation (electroporation)***

1μL of crude yeast plasmid DNA was combined with 25μL of electrocompetent HB101 and transferred to an electroporation cuvette (0.2cm electrode gap). The electroporator was set at 200Ω, 25μF and 1.5kV. 400μL of M9 minimal media was

added to the transformed HB101 and bacteria were allowed to recover for 1 hour at 37°C with moderate agitation. 200μL of this mixture was added to M9 minimal media AMP plates to nutritionally select for HB101 containing library plasmids.

2.2.8 Maintenance and growth of yeast

Y190 and HF7c were grown at 30°C using YEP(D) media with the addition of glucose to a final concentration of 2% as described (Ausubel *et al.*, 1994). Yeast transformed with plasmids containing auxotrophic markers were grown in synthetic complete (SC) media consisting of minimal essential components lacking the appropriate amino acids for selection of the plasmid.

2.2.9 Yeast 2-hybrid screen

2.2.9.1 *Large scale yeast transformation for screening*

Large scale yeast transformations were performed as described previously (Schiestl *et al.*, 1993). HF7c previously transformed with the bait plasmid, pGBT9-HCF-$1_{450\text{-}1439}$, were grown in 50mL of SC media lacking tryptophan (to select for yeast containing pGBT9) at 30°C overnight to saturation. The following day this culture was transferred to 500mL of prewarmed SC(-Leu) media and grown at 30°C with shaking until the cell density reached $2x10^7$ cells/mL. All equipment and solutions were subsequently kept ice cold and yeast were maintained on ice. Cells were harvested three times by centrifugation for 10 minutes at 3000 x g and sequentially resuspended in 200mL of sterile water, 10mL of sterile water, and finally 4.5mL of 100mM LiAc. Yeast resuspended in LiAc were then incubated for 15 minutes at 30°C. Added to this was 5mL of sonicated salmon testes DNA (1mg/mL) previously boiled for 10 minutes and

flash chilled on ice, 24mL of 50% w/v PEG-3350, 3.6mL of 1M LiAc, 2mL of sterile water and 100µg of Clontech MatchMaker HeLa cDNA plasmid library. This mixture was vortexed briefly and incubated for 30 minutes at 30°C, followed by a heat shock of 30 minutes at 42°C, and incubated at room temperature for one hour. Finally, the yeast cells were harvested and resuspended in 10mL of sterile water and 500µL of this mixture was plated onto twenty SC plates lacking leucine, tryptophan, (to select for yeast containing the bait plasmid which contains the tryptophan marker, and the library plasmid which contain a leucine marker) and histidine (to select for interactions) and containing 20mM 3-amino-1,2,4-triazole. In addition, 1µL of cells was plated onto 2 SC plates lacking leucine and tryptophan only to determine the total number of transformants. Two million transformants were screened by this method and library plasmids (containing a leucine marker) from His^+ colonies were selectively recovered from yeast by transformation into *Escherichia coli* HB101 ($leuB^-$).

2.2.9.2 *Isolation of library plasmids from His^+ Yeast*

Library plasmid isolation from HF7c was performed by preparing a crude yeast DNA extract as described (Ausubel *et al.*, 1994) and transforming *E. coli* HB101 ($leuB^-$) to selectively isolate library plasmids. Briefly, His^+ HF7c containing the plasmid of interest were grown in 5mL SC lacking leucine overnight at 30°C to saturation. The following day 1.5mL of the culture was pelleted in a microcentrifuge tube and resuspended in 200µL of breaking buffer (2% v/v Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl pH 8.0, and 1mM EDTA) to which was added ~0.3g of acid-washed glass beads and 200µL of phenol/choloform. The mixture was vortexed at high speed for

2 minutes and spun for 5 minutes at high speed at room temperature. The aqueous layer was retained and 1μL was used to transform electrocompetent HB101. Transformed HB101 (*leuB*$^-$) were grown on M9 minimal media (Sambrook *et al.*, 1989) for nutritional selection of HB101 bacteria complemented by library plasmids containing a leucine marker. Plasmids isolated from bacterial colonies that grew on M9 plates by the method described were subjected to restriction enzyme analysis with *Eco*RI and *Xho*I to identify library plasmids since *Eco*RI and *Xho*I were unique sites in the library plasmids flanking the cDNA inserts.

2.2.10 Yeast 2-hybrid assay

2.2.10.1 *Small scale yeast transformations*

Small scale yeast transformations were performed by the lithium acetate method as described (Elble, 1992). Yeast were grown to saturation at 30°C with moderate agitation, transferred to microcentrifuge tubes and pelleted by centrifugation at 8000 rpm for 2 minutes. The supernatant was removed and to the yeast pellet was added 10μg of salmon testes DNA, 1-2μg of plasmid, and 500μL of PLATE solution (40% PEG-3350, 100mM LiAc, 10mM Tris-HCl pH 7.5, and 1mM EDTA). The transformations were left at room temperature overnight. The following day, the yeast was pelleted by centrifugation at 8000 rpm for 2 minutes and the PLATE solution was removed. The yeast pellet was resuspended in 200μL of SC media lacking leucine and tryptophan and added to plates lacking the appropriate amino acid(s) to allow for selection of yeast harbouring the appropriate plasmids. Plates were incubated at 30°C for 3-4 days until colonies appeared.

2.2.10.2 *Qualitative β-galactosidase overlay assay*

An interaction between two proteins is indicated in the yeast 2-hybrid assay by the increased expression of a reporter gene which contains an upstream activating sequence recognized by GAL4DBD. The *lacZ* gene is integrated into the genome of Y190 yeast and serves as such a reporter. A qualitative assessment of the interaction can be obtained by the β-galactosidase agarose overlay assay (Bohen and Yamamoto, 1993), which is based on the cleavage of a chromogenic substrate (X-gal) by β-galactosidase on immobilized yeast colonies, resulting in blue colour formation. Briefly, 0.5% agarose was dissolved in 0.5M $NaPO_4$ pH 7.0 buffer, to which was added SDS to a final concentration of 0.1%, and X-gal to a final concentration of 0.4mg/mL dissolved in dimethyl formamide. The solution was allowed to cool to ~ 50°C, added directly onto yeast growing on solid medium, and placed at 37°C. The appearance of blue colour, indicative of β-galactosidase activity, was monitored.

2.2.10.3 *Quantitative Liquid β-galactosidase Assay*

To quantify lacZ activity, an assay measuring the lacZ activity of yeast growing in liquid culture as described previously (Ausubel *et al.*, 1994) was performed. Briefly, individual yeast colonies were grown to saturation overnight at 30°C with moderate agitation. The following day, 500μL of the overnight culture was diluted into 3mL of fresh media and grown to OD_{600} ~1.0. Cells were transferred to microcentrifuge tubes and harvested by centrifugation at 8000 rpm for 2 minutes at room temperature. The supernatant was removed and cells resuspended in 1mL of Z buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, and 50mM β-mercaptoethanol, adjusted to

pH 7.0) and the OD_{600} was determined for later calculations. 200µL of the resuspension was diluted into 800µL of fresh Z buffer in duplicate and the yeast was lyzed by 3 successive freeze/thaw cycles in liquid nitrogen. To this was added 0.2mL of 4mg/mL o-nitrophenyl-β-D-galactoside, (ONPG), the lacZ substrate, which is a chromogenic substrate that produces a yellow colour when cleaved by lacZ, allowing absorbance measurements at OD_{420}. The reaction was incubated at 30°C until yellow could be seen, at which point the reaction as terminated by addition of 300µL of 1M Na_2CO_3. The samples were centrifuged at high speed for 1 minute to pellet cellular debris and the supernatants were transferred to cuvettes for spectrophotometric analysis. β-galactosidase activity units were determined using the following formula:

$$U = 1000 (OD_{420} - 1.75 \times OD_{550}) / t \times v \times OD_{600}$$

U = units of β-galactosidase activity
OD_{420} = o-nitrophenyl absorbance and light scattering by cell debris
OD_{550} = light scattering by cell debris
OD_{600} = cell density
t = reaction time (minutes)
v = volume (mL)

2.2.11 GST pull-down assay

Protein binding assays with GST fusion proteins, and ^{35}S-methionine radiolabelled proteins synthesized *in vitro* using a coupled rabbit reticulocyte transcription/translation system (Promega) were carried out as described previously (Meertens *et al.*, 1998). Briefly, *E. coli* BL21(DE3) harbouring GST expression vectors were grown to an OD_{600} of 0.6-0.8 and induced with 0.1mM isopropylthiogalactosidase (Biosynth AG) for 3 hours. Bacteria were collected by centrifugation and re-suspended

in NETN buffer (0.5% NP40, 1mM EDTA, 20mM Tris-Cl pH 8.0, 100mM NaCl, and 1 tablet mini C protease inhibitor (Boehringer Mannheim)/25mL buffer) and cell extracts were prepared by sonication. 50µl of a 50:50 slurry of glutathione-Sepharose 4B was incubated with clarified cell extracts containing GST or GST-fusion protein for 1 hour at 4°C. Beads were collected by centrifugation, washed twice with phosphate buffered saline, and beads containing equivalent amounts of bound protein (as determined by Commassie blue staining of sodium dodecyl sulphate (SDS)-polyacrylamide gels) were incubated with 10-20µl of reticulocyte lysate containing radiolabelled translated protein in IPAB buffer (150mM KCl, 0.02 mg/mL bovine serum albumin, 0.1% Triton X-100, 0.1%NP40. 5mM $MgCl_2$, and 20mM Hepes pH 7.9) for 2-3 hours at 4°C. Beads were washed extensively with IPAB buffer lacking bovine serum albumin and bound radiolabelled proteins were eluted from the beads by boiling in SDS sample buffer and analysed by SDS-polyacrylamide gel electrophoresis.

GST pull-down assays were also employed to determine competitive binding. In these asssays, the procedure was performed as described above except that an unlabelled competitor protein, produced in *vitro* using a coupled rabbit reticulocyte transcription/translation system (Promega), was incubated in the presence of the radiolabelled protein. In these assays the amount of rabbit reticulocyte lysate was kept constant by adding unprogrammed lysate to the binding reactions as required.

2.2.12 *In vitro* transcription/translation reactions

In vitro transcription/translation reactions using rabbit reticulocyte lysates were performed according to the manufacturer's instructions (Promega). Proteins were

synthesized in the presence of ^{35}S-methionine to generate radiolabelled proteins which could be visualized by autoradiography. Briefly, radiolabeled proteins were produced in 50μL reactions containing 25μL TNT rabbit reticulocyte lysate, 2μL TNT reaction buffer, 1μL SP6 or T7 RNA polymerase, 1μL amino acid mixture lacking methionine, 10μL ^{35}S-methionine, 1μL RNA guard, and 1μg of DNA template. The reactions were incubated at 30°C for 90 minutes.

2.2.13 Cell Culture Maintenance

COS-1 cells were maintained at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. tsBN67$_{HR1}$ cells were cultured at 33.5°C in Dulbecco's modified Eagle medium supplemented with 10% calf serum, 1% L-glutamine, and 1% penicillin-streptomycin.

2.2.14 Transient transfections

Cells were seeded in 6 well plates at a concentration of 2-3 x 10^5 cells/well one day prior to transfection to achieve 70-80% confluence on the day of transfection. Transfections were carried out using the LipofectAMINE reagent as outlined by the manufacturer (Invitrogen). Total DNA and promoter dosage was kept constant with the appropriate amounts of corresponding empty vectors. Briefly, up to 2μg of total plasmid DNA and 8μL of lipofectamine reagent were incubated in 400μL of unsupplemented DMEM for 30 minutes. Cells in 6 well plates were washed with PBS and 1mL of unsupplemented DMEM was added to each well during the 30 minute incubation. Following the incubation, 1.6mL of unsupplemented DMEM was added to the 400μL

mixture and the resulting 2mL was mixed and split into 1mL per well for duplicates of each transfection. The cells were incubated with the transfection mixture for 4-5 hours before it was replaced with fresh DMEM media supplemented with 10% serum, 1% L-glutamine, and 1% penicillin-streptomycin. The media was also replaced the following day and the cells harvested on the second day post-transfection.

2.2.15 Luciferase assay

A luciferase gene was used in transient transfections (deWet *et al.*, 1987). The following method was used to quantify luciferase gene expression. On the second day post-transfection, cells were harvested by washing twice with PBS, followed by a 10 minute incubation with 400μL of 1X reporter lysis buffer (Promega). Samples were removed from the plate using a cell lifter, collected in microcentrifuge tubes and pelleted by centrifugation for 2 minutes at high speed. Supernatants were collected and assayed for luciferase activity using a Lumat LB9507 luminometre (PerkinElmer, Life Sciences) according to the manufacturer's instructions. Briefly, 10μL of extract was placed in a 13x100mm plastic tube (Sarstedt) and placed within the luminometre, programmed to inject 100μL of luciferin reagent (470 μM luciferin, 270μM coenzyme A, 530μM ATP, 33μM DTT, 20mM Tricine, 1.07mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, and 0.1 mM EDTA) to each sample. The resulting luminescence reflected the luciferase activity present in the sample and was recorded as relative light units (RLUs) which were normalized to the total protein concentration in each sample using the BioRad protein assay according to the manufacturer's instructions (BioRad).

2.2.16 Co-immunoprecipitation assay

For the Miz-1/HCF-1 co-immunoprecipitation assays, COS-1 cells were transfected as described in a previous section. However, for the E2F-4/HCF-1 co-immunoprecipitation assays the transfections were performed with different cells and on a larger scale. Specifically, $tsBN67_{HR1}$ cells were seeded onto 10cm diameter plates at a concentration of 2-3 x 10^6/plate 1 day prior to transfection to achieve 70-80% confluence. Transfections were carried out using LipofectAMINE reagent (50μL/plate; Invitrogen) as outlined by the manufacturer with a total of 10μg of indicated plasmids. Extracts were prepared 48 hours post-transfection using 1mL of NP40 buffer (50mM Tris pH 7.5, 150mM NaCl, 1%NP40 and 0.2mM PMSF). Approximately 800μL of supernatant, normalized for protein concentration, was incubated with 1-2μg of antibody for 2-24hrs, followed by a 2-8hr incubation with 50μL of protein G-Sepharose at 4°C. Immune complexes were collected, extensively washed, suspended in 50μL of 2X SDS-polyacrylamide gel sample buffer, and subjected to PAGE. Proteins were transferred to Hybond™-C pure nitrocellulose membrane (Amersham Life Sciences) and probed using appropriate primary antibody. Primary antibody addition was followed by horseradish peroxidase-coupled secondary antibody (Amersham Life Sciences). Proteins were detected by enhanced chemiluminescence with a commercially available kit (ECL, Amersham Life Sciences) according to the manufacturer's instructions.

2.2.17 Western Blotting

COS-1 and $tsBN67_{HR1}$ cells were transfected as described above for "transient transfection", except that 1-2μg of the various expression plasmids was used. Two days

post-transfection, cells were harvested for Western blot analysis. Cells were washed twice with PBS and harvested with 300μL of RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.1% SDS, 1% sodium deoxycholate, and 1mM PMSF). Samples were collected to microcentrifuge tubes, sonicated once for 30 seconds, and pelleted by centrifugation at high speed for 1 minute at 4°C to remove cellular debris. The supernatants were collected and protein concentration of each sample was determined using a BioRad protein assay kit. Equivalent amounts of total protein and pre-stained molecular weight markers were resolved by SDS-PAGE. Proteins were transferred to Hybond^{TM}-C pure nitrocellulose membrane (Amersham Life Sciences) overnight at 70mA at 4°C. Membranes were blocked the following morning using 4% skim milk powder in TBST buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% Tween 20) for 1 hour and then washed twice using TBST buffer for 15 minutes. Subsequently, an appropriate dilution of primary antibody (diluted in TBST containing 1% milk powder) was used to probe the membrane for 1 hour. The membrane was washed twice with TBST as before, and incubated with an appropriate dilution of HRP-conjugated secondary antibody (Amersham Life Sciences) (diluted in TBST containing 0.2% milk powder) for 1 hour. The membrane was once again washed twice with TBST as before prior to antibody detection with a commercially available ECL kit (Amersham Life Sciences) as instructed. The membrane was then exposed to X-OMAT Blue XB-1 film (Kodak) for up to sixty minutes and developed.

2.2.18 tsBN67$_{HR1}$ complementation assay

tsBN67$_{HR1}$ cells were seeded with 8 x 10^5 cells in 6cm diametre plates and grown at 33.5°C one day prior to transfection to achieve 70-80% confluence. Cells were transfected the next day using LipofectAMINE reagent (20μL/plate) (Invitrogen). 48 hours later, cells were trypsinized and re-suspended in 5mL of media supplemented with 800μg/mL G418 (Gibco). The suspension was divided in two, and used to seed 2 10cm diameter plates and maintained under selection with G418 for 21 days at either 33.5°C or 40°C. The media was replaced with fresh media every 3-4 days. After 21 days, cells were washed with 10mL of PBS and stained with 0.1% crystal violet.

2.2.19 BLAST search for HBM-containing cellular proteins

To identify cellular proteins that may interact with HCF-1 we utilized the BLAST search algorithm and a degenerate HBM sequence to identify cellular proteins that contain the HCF-binding motif (HBM). This short motif (D/EHxY) generated a large number of potential HCF-1 interacting proteins. We focused on proteins with a known role in cell cycle control as the most likely to be *bona fide* HCF-1 interaction partners.

CHAPTER THREE

RESULTS

3.1 IDENTIFYING MIZ-1 AS A NOVEL HCF-1 BASIC DOMAIN INTERACTION PARTNER AND CHARACTERIZING ITS INTERACTION

3.1.1 Yeast 2-hybrid screening reveals Miz-1 to be an HCF-1 interaction partner

HCF-1 is a multifunctional protein that promotes cellular proliferation, though a clear understanding of the mechanisms of HCF-1-mediated cellular proliferation has not been elucidated (Goto *et al.*, 1997; Wilson *et al.*, 1997; Julien and Herr, 2003). In order to identify novel HCF-1-interaction partners that may provide insights into the cellular functions and mechanisms of action of HCF-1, we initially performed yeast 2-hybrid screens (Fields and Song, 1989) which have a powerful application as a genetic screen to identify interaction partners for a candidate bait protein due to the modular nature of transcription factors. Similar approaches by others have focused on the N-terminal kelch domain of HCF-1 since this domain contains the P134S mutation which causes cell cycle arrest at the non-permissive temperature in tsBN67 cells and thus is expected to interact with key cell cycle regulatory proteins (Freiman and Herr, 1997; Lu *et al.*, 1997; Mahajan *et al.*, 2002). However, we focused on a separate region of HCF-1 (residues 450-1439) that encompasses the basic domain, since this region has been shown to be required for HCF-1-mediated cell cycle progression in cooperation with the N-terminal kelch domain (Wilson *et al.*, 1997). It was hypothesized that the basic domain would associate with cellular proteins that play a role in cell cycle regulation by HCF-1 that are independent of the kelch domain.

Yeast 2-hybrid screens were performed using the Clontech Matchmaker system according to the manufacturer's instructions. In the system used here, a fusion of HCF-1 encompassing the basic domain (HCF-$1_{450-1439}$) was made to GAL4DBD to act as the bait in the screen while a HeLa cell cDNA library fused to GAL4AD was used as the prey. Yeast strain HF7c containing pGBT9-HCF-$1_{450-1439}$ was transformed with a HeLa cell Matchmaker cDNA library fused to GAL4 activation domain (Clontech) by the lithium acetate method (Elble, 1992). 2 x 10^6 independent transformants were grown on his^-, leu^-, and trp^- plates supplemented with 20mM 3-amino-1,2,4-triazole. Library plasmids from His^+ colonies were selectively recovered from yeast following transformation into *E. coli* HB101 (*leuB*$^-$), transformed along with pGBT9-HCF-$1_{450-1439}$ or control GAL4-DBD plasmids into yeast strain Y190 and assayed for β-galactosidase activity using the β-galactosidase overlay assay (Ausubel *et al.*, 1994). Colonies that scored positive for β-galactosidase activity in the presence of the HCF-1 bait plasmid but not with control or irrelevant plasmids were sequenced. Among the clones isolated was a partial cDNA encoding amino acids 269-803 of Miz-1, a zinc-finger transcription factor, previously identified as an interaction partner for the cellular oncogene c-myc (Peukert *et al.*, 1997), in addition to a partial cDNA encoding the carboxy-terminus of an uncharacterized protein we have tentatively termed HIP-2, HCF-1-interaction partner-2. Thus, by performing a yeast 2-hybrid screen, we had identified 2 novel HCF-1 interaction partners. Miz-1 is an 803 amino acid protein containing 13 zinc fingers of the C_2H_2 class, in which pairs of cysteines and histidines are separated by a loop of twelve amino acids (Evans and Hollenberg, 1988; Peukert *et al.*, 1997; Laity *et al.*, 2001), and an N-terminal POZ

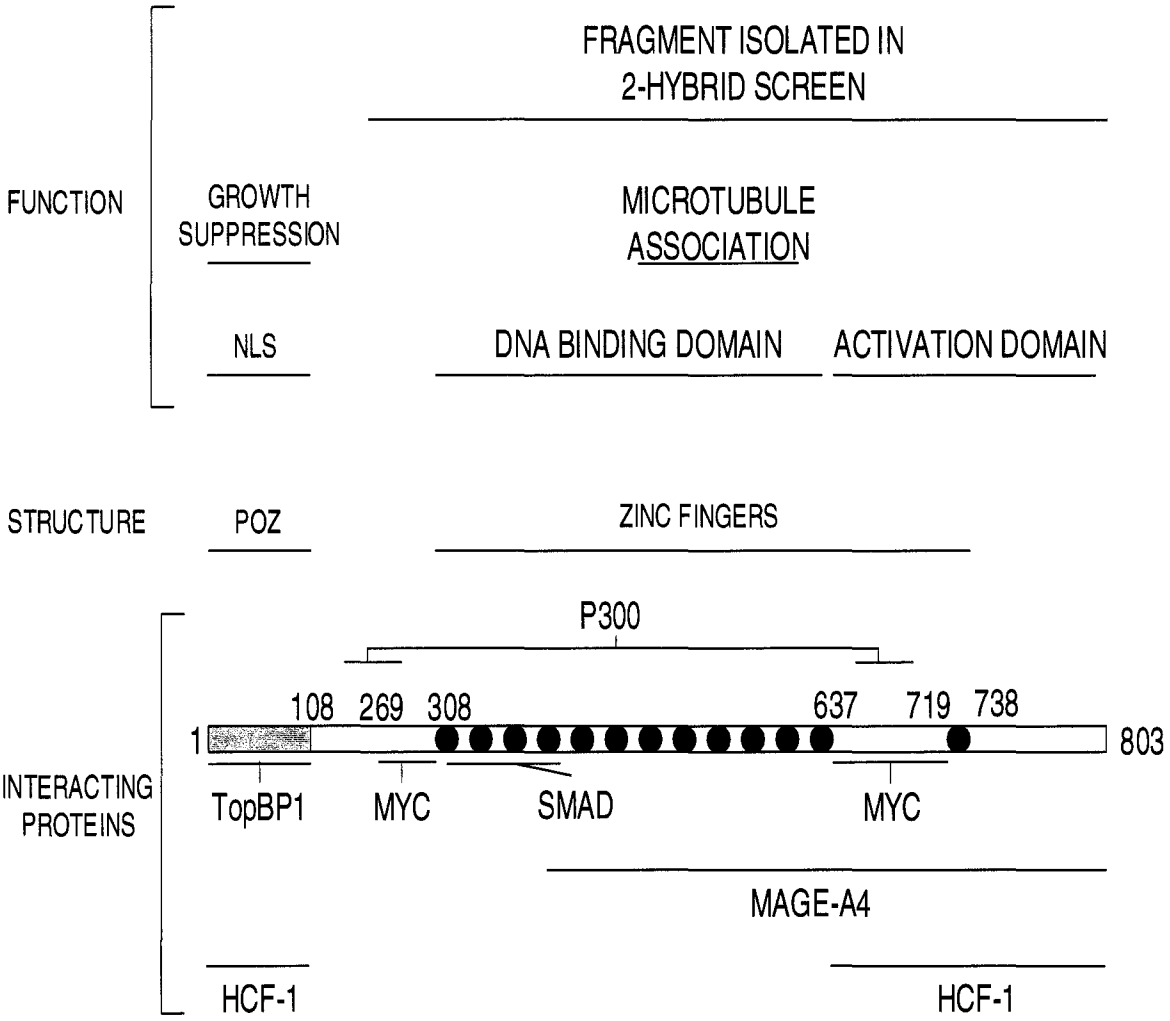

Figure 3.1: Schematic representation of Miz-1. The modular domain structure of Miz-1 is shown indicating the N-terminal POZ domain and the 13 zinc finger motifs (represented by ovals) and including the various functions and interacting partners associated with each domain. Also illustrated is the region of Miz-1 (residues 269-803) encoded in the cDNA retrieved in the 2-hybrid screen.

domain (figure 3.1; Peukert *et al.*, 1997). Miz-1, originally identified through 2-hybrid screening for c-myc-interacting proteins (Peukert *et al.*, 1997), is an integral part of the anti-mitogenic TGF-β signaling pathway and also contributes to cell cycle arrest following DNA damage and differentiation signals (Seoane *et al.*, 2001; Herold *et al.*, 2002; Seoane *et al.*, 2002; Wu *et al.*, 2003; Zhao *et al.*, 2004). It stimulates transcription by binding directly to the 5' regulatory region, often at initiator elements (Inr), of target genes encoding cell cycle inhibitory proteins (Peukert *et al.*, 1997; Seoane *et al.*, 2001; Staller *et al.*, 2001; Seoane *et al.*, 2002; Kime *et al.*, 2003; Wu *et al.*, 2003; Barsyte-Lovejoy *et al.*, 2004). Subsequent studies have shown that Miz-1 is required for early embryonic development as Miz-1 deficient embryos succumb to apoptosis of ectodermal cells early in embryonic development, an outcome which may result in part due to the absence of $p57^{Kip2}$ expression in the Miz-1 deficient embryo (Adhikary *et al.*, 2003). Paradoxically, recent results using small interfering RNA duplexes (siRNAs) to ablate Miz-1 expression demonstrated that Miz-1 may be required for cellular proliferation (Ziegelbauer *et al.*, 2004).

In addition to Miz-1, our yeast 2-hybrid screen also revealed that a second, uncharacterized, protein interacts with HCF-1, which we have tentatively named HIP-2. The HIP-2 cDNA isolated from the yeast 2-hybrid screen contains the carboxy-terminal portion of the polypeptide as we located a stop codon and a polyA tail sequence at its 3' end, however it lacks an identifiable translational start codon, indicating that we have isolated a partial cDNA for a novel protein which interacts with HCF-1. The sequence of the HIP-2 cDNA is shown in figure 3.2. HIP-2 possesses a unique sequence of nine

```
cagGAATTCggcacgagtgcagctccagcccctccgaccagccccagaagtaaccacggtccagctccagc
          A  R  V  Q  L  Q  P  L  R  P  A  P  E  V  T  T  V  Q  L  Q

cagcgcaggaggtgaccacagtccagctccagccagcacaggaagtaaccacggtccagctccagccagca
P  A  Q  E  V  T  T  V  Q  L  Q  P  A  Q  E  V  T  T  V  Q  L  Q  P  A

caggaggtgaccacggtccagctccagcccgtggccggccagctctccaattccagtgggggagctgtggc
 Q  E  V  T  T  V  Q  L  Q  P  V  A  G  Q  L  S  N  S  S  G  G  A  A  A

tactgaggcacccaacctgctggttgttcagagcggggcagctgaggagttgctcactggcccgggccccg
  T  E  A  P  N  L  L  V  V  Q  S  G  A  A  E  E  L  L  T  G  P  G  P

gggaggcgggggatggcgaggccagcactggtgtggtccaggatgtcctctttgagacactccagacggac
G  G  A  G  D  G  E  A  S  T  G  V  V  Q  D  V  L  F  E  T  L  Q  T  D

gagggcttgcagagcgtgctggtgctgagcggggccgatggcgaacagactcgactctgcgtacaggaggt
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gtggtccccgcaggagctgggcctggtgttatgacccctcagggcctgccctccatccagattgtccagac
 V  V  P  A  G  A  G  P  G  V  M  T  P  Q  G  L  P  S  I  Q  I  V  Q  T

tctacccgcagtccagctggtgcacacgtttTGAggagaggcagtgattcccctcccgccccgcacagaga
  L  P  A  V  Q  L  V  H  T  F STOP

ccccaactcactgccagccggggcggggcagggtgccgcaggctgggcttgctaataaagacccgagtctc

cccAAAAAAAAAAAAAAAAAAAAAActcgaggg
```

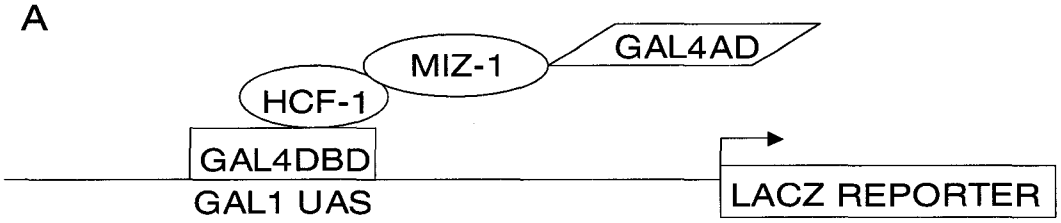
Figure 3.2: HIP-2 nucleotide and amino acid sequence. The nucleotide sequence (shown in lower case letters) of HIP-2 displays the cDNA obtained through yeast 2-hybrid screening for HCF-1 interaction partners from a HeLa cell cDNA library. The EcoRI and XhoI sites (shown in italics) mark the 5' and 3' ends of the sequence as these were the sites used in constructing the library. The stop codon and polyA tail (shown in bold) indicate that the HIP-2 cDNA is complete at the 3' end. The amino acid sequence (shown in upper case letters) reveals a unique sequence of nine amino acids, EVTTVQLQP, repeated four times (shown underlined).

amino acids, EVTTVQLQP, repeated four times which we have been unable to find in any other protein currently in the protein databases, and for which we do not know the function. Further downstream of the repeat elements, Helen Wong has determined that HIP-2 possesses a region that functions as a transcriptional activation domain, in addition to serving as the HCF-1 interaction surface (Wong, 2002). Although both Miz-1 and HIP-2 were intriguing factors that may help to characterize the function of HCF-1 in the cell, I chose to focus on Miz-1 due to its known role in cell cycle control.

3.1.2 Validating and confirming 2-hybrid results

The yeast 2-hybrid screen has been used as a valuable tool to identify novel interaction partners for a large number of proteins. However, the assay is known to produce false positive results. With this in mind, before proceeding to further characterize the novel HCF-1/Miz-1 interaction we had discovered via 2-hybrid screening, we eliminated the possibility of a false positive for this interaction by performing several control experiments with the yeast 2-hybrid assay and confirmed it using an in vivo co-immunoprecipitation assay (figure 3.3). As shown in figure 3.3b, in the yeast 2-hybrid assay HCF-1 bound specifically to Miz-1 as β-galactosidase activity was observed only in yeast transformed with both GAL4DBD-HCF-1 bait and GAL4AD-Miz-1 prey plasmids. Significantly, Miz-1 failed to interact with the HCF-1 kelch domain (residues 1-380), a domain that is sufficient to interact with VP16 (Simmen *et al.*, 1997; Wilson *et al.*, 1997) and a number of cellular factors (Freiman and Herr, 1997; Lu *et al.*, 1997; Lu and Misra, 2000; Lin *et al.*, 2002; Mahajan *et al.*, 2002a; Luciano and Wilson, 2003), but absent from the 2-hybrid bait.

A



B

GAL4 DBD FUSION	GAL4 AD FUSION	INTERACTION
HCF-$1_{450-1439}$	-	-
HCF-$1_{450-1439}$	GAL4AD	-
-	MIZ-$1_{269-803}$	-
GAL4DBD	MIZ-$1_{269-803}$	-
HCF-$1_{450-1439}$	MIZ-$1_{269-803}$	++
HCF-1_{1-380}	MIZ-$1_{269-803}$	-
HCF-1_{1-380}	VP16	++

C

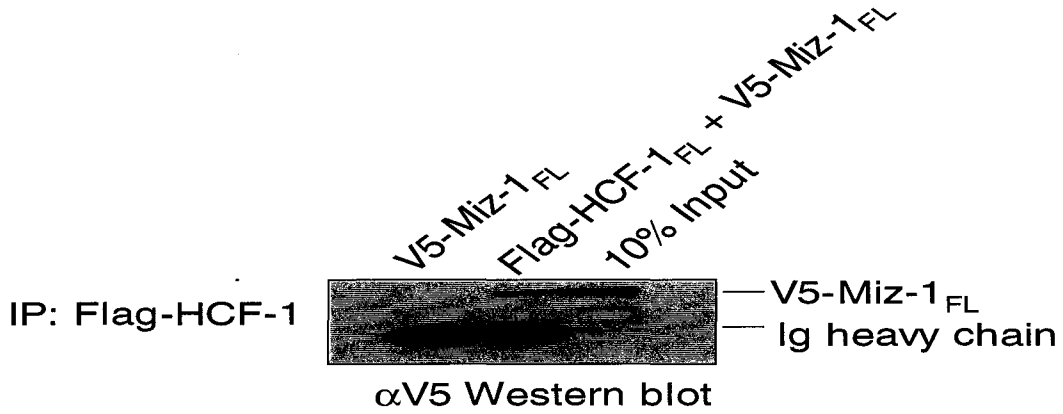


Figure 3.3: HCF-1 interacts with Miz-1. A. Schematic diagram of the yeast 2-hybrid assay. The modular nature of the GAL4 transcription factor allows for the reconstitution of GAL4 expressed from separate GAL4DBD-HCF-1 and GAL4AD-Miz-1 fusions as a result of the non-covalent HCF-1/Miz-1 assocation. B. Two-hybrid analysis of the HCF-1/Miz-1 interaction. Yeast strain Y190 harbouring the indicated GAL4 DBD and GAL4 activation domain fusions (numbering corresponds to the amino acids in the respective proteins) were assayed for ß-galactosidase expression by the X-gal overlay assay method. The GAL4-VP16 activation domain fusion plasmid contained residues 1-404 of VP16. Interaction is indicated by (++). C. HCF-1 and Miz-1 interact *in vivo* in mammalian cells. COS-1 cells were transfected with expression vectors for FLAG-HCF-1_{FL} and V5-Miz-1 $_{FL}$ or V5-Miz-1 $_{FL}$ alone as indicated. Cell lysates were immunoprecipitated (IP) with anti-FLAG and the precipitates resolved on a SDS-polyacrylamide gel probed with anti-V5 antibody to detect Miz-1. 10% input represents 10% of the lysate used in the immunoprecipitation reactions.

To determine whether HCF-1 and Miz-1 form a complex *in vivo* in mammalian cells, COS-1 cells were transfected with expression vectors encoding V5-tagged full length Miz-1 and FLAG-tagged full length HCF-1. Protein extracts were immunoprecipitated with anti-FLAG antibody, resolved on a SDS-polyacrylamide gel, and probed with anti-V5 antibody (figure 3.3c). Miz-1 was detectable in immune complexes precipitated with anti-FLAG antibody from cells which had been transfected with both Flag-HCF-1_{FL} and V5-Miz-1_{FL} but not from control cells which were transfected with V5-Miz-1_{FL} alone. Thus, co-immunoprecipitation of V5-Miz-1_{FL} was dependent on co-expression of FLAG-HCF-1_{FL}, indicating that HCF-1 and Miz-1 interact within extracts generated from mammalian cells.

3.1.3 HCF-1 interacts with Miz-1 via determinants in the basic domain

The bait used in the yeast 2-hybrid screen contained both the basic domain and the HCF_{PRO} repeats. To gain insight into the functional relevance of this interaction we sought to map more precisely the region in HCF-1 targeted by Miz-1. To this end, we constructed a series of deletions within GAL4-DBD-HCF-$1_{450\text{-}1439}$ and tested these for interaction by two-hybrid analysis with GAL4-AD-Miz-$1_{269\text{-}803}$ (see figure 3.4). Deletion of the HCF_{PRO} repeats did not influence binding, indicating that these residues do not contribute to interaction with Miz-1. In contrast, the basic domain (residues 450-902) was sufficient for Miz-1 interaction. Furthermore, a fragment of HCF-1 spanning residues 750-836 was sufficient to bind to Miz-1. However, strongest binding, as reflected by β-galactosidase activity, was observed with HCF-$1_{750\text{-}902}$, suggesting that residues between 836 and 902 may contribute to more robust binding and/or stability of

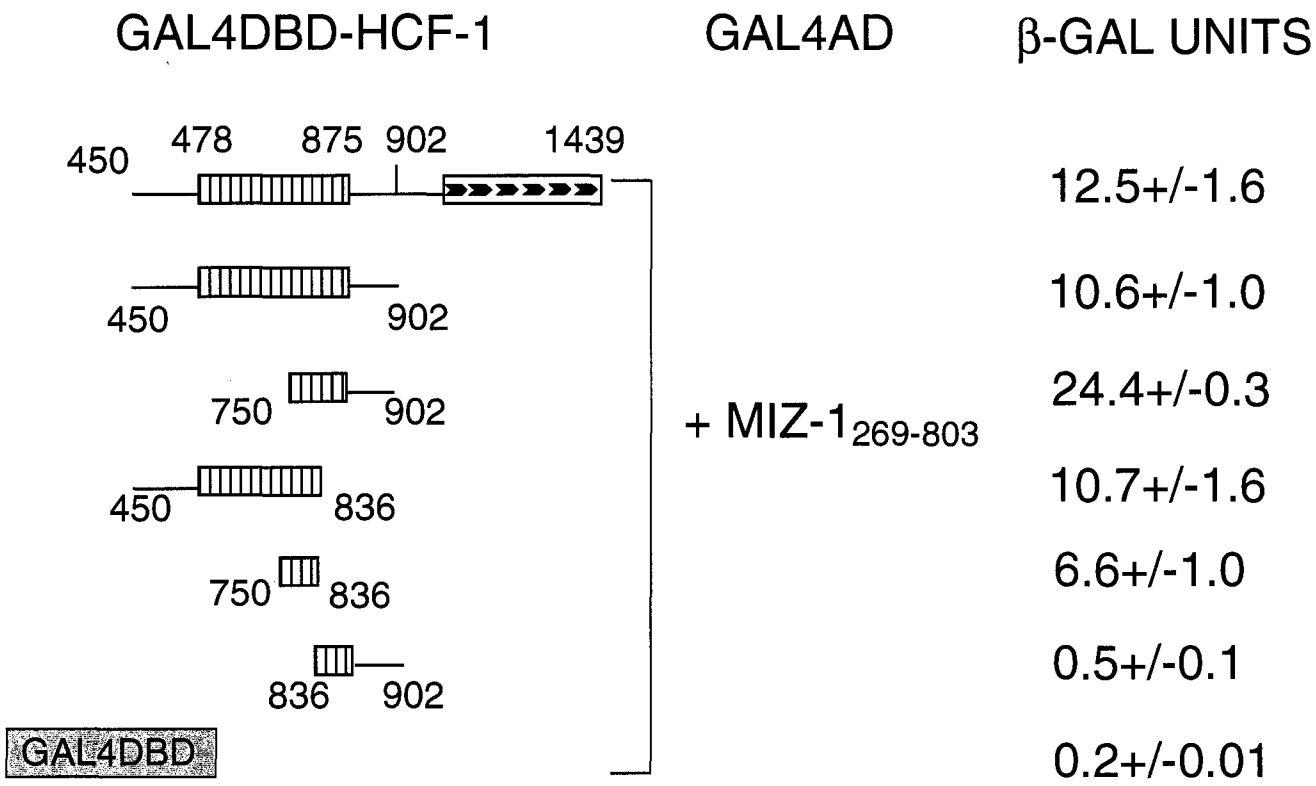


Figure 3.4: Miz-1 targets the basic region of HCF-1. Amino- and carboxy-terminal deletions of HCF-1 between residues 450 to 1439, as indicated, were cloned into the GAL4-DBD plasmid and tested for interaction with Miz-1 (residues 269-803) by two-hybrid analysis in yeast. Specific ß-galactosidase activity was determined by the liquid quantitative assay as outlined in the methods section. The values shown represent the average ß-galactosidase activity (+/- SD) from three independent transformants assayed in duplicate.

the HCF-1/Miz-1 complex *in vivo*. A fragment spanning residues 836-902 was, however, insufficient to bind with Miz-1. In order to confirm this novel interaction and determine whether Miz-1 binds directly to HCF-1, we conducted *in vitro* GST pull-down assays with radiolabelled proteins synthesized *in vitro* (see figure 3.5). As shown in the figure, radiolabelled full length Miz-1 bound to GST-HCF-$1_{750\text{-}902}$ but not to GST alone. Thus, taken together, these results indicate that Miz-1 directly targets part of the basic domain of HCF-1, a domain that is required for cell cycle progression (Wilson *et al.*, 1997), with amino acids between 750-836 being of particular importance for interaction.

3.1.4 HCF-1 targets two separate domains of Miz-1

Miz-1 interacts with a variety of cellular proteins, including c-myc (Peukert *et al.*, 1997), p300 (Staller *et al.*, 2001), SMAD proteins (Seoane *et al.*, 2001), TopBP1, (Herold *et al.*, 2002), and MAGE-A4 (Sakurai *et al.*, 2004) as summarized in figure 3.1. The c-myc/Miz-1 interaction is mediated by two separate regions in Miz-1 with fragments spanning residues 269-308 and 637-718 necessary for interaction (Peukert *et al.*, 1997). Interestingly, determinants overlapping these separate regions are also important for interaction with the transcriptional co-activator, p300 (Staller *et al.*, 2001). The SMAD/Miz-1 interaction is mediated via a distinct region that encompasses the initial 4 amino-terminal zinc fingers (Seoane *et al.*, 2001), whereas TopBP1 targets the Miz-1 POZ domain for interaction (Herold *et al.*, 2002). To identify the region(s) of Miz-1 that are involved in interaction with HCF-1, and potential relationships to other protein-protein interaction surfaces present in Miz-1, we generated a series of deletions of Miz-1 and tested binding to HCF-1 using *in vitro* GST pull-down assays. As shown in figure

3.5a, a radiolabelled Miz-1 C-terminal fragment spanning residues 637-803 bound efficiently and specifically to GST- HCF-$1_{750-902}$ similar to the efficiency with which radiolabelled full length Miz-1 bound GST-HCF-$1_{750-902}$. In addition, in reciprocal binding experiments, radiolabelled HCF-$1_{612-902}$ bound to GST-Miz-$1_{637-803}$. Interestingly, the carboxy-terminal region of Miz-1 which binds HCF-1 also contains determinants that mediate binding with c-myc and p300. Specifically, the c-myc/Miz-1 and p300/Miz-1 interaction surfaces have been mapped to residues 637-718 (Peukert *et al.*, 1997) and 683-715 (Staller *et al.*, 2001), respectively, within the carboxy-terminal region of Miz-1. C-Myc and p300 also target amino- terminal residues 269-308 and 190-294 of Miz-1, respectively (Peukert *et al.*, 1997; Staller *et al.*, 2001). However, as shown in figure 3.5a, radiolabelled HCF-$1_{612-902}$ was unable to bind to overlapping sub-fragments of Miz-1 corresponding to residues 637-738 or 719-803. These results suggest that the Miz-1/HCF-1-interaction is mediated by multiple determinants, or an extended region within residues 637-803 of Miz-1, and that HCF-1 targets determinants in Miz-1 that overlap with, but are distinguishable from, those required for interaction with c-myc, p300, and SMAD proteins.

The Miz-1 fragment isolated in the 2-hybrid screen encoded residues 269-803, thus it lacked the amino-terminal residues which encompass the POZ domain (residues 1-108). This indicates that the POZ domain is not required for interaction with Miz-1, at least in the presence of the carboxy-terminal interacting domain. However, since POZ domains have been shown to provide protein interaction interfaces (Bardwell and Treisman, 1994; Albagli *et al.*, 1995; Collins *et al.*, 2001), we tested whether this region

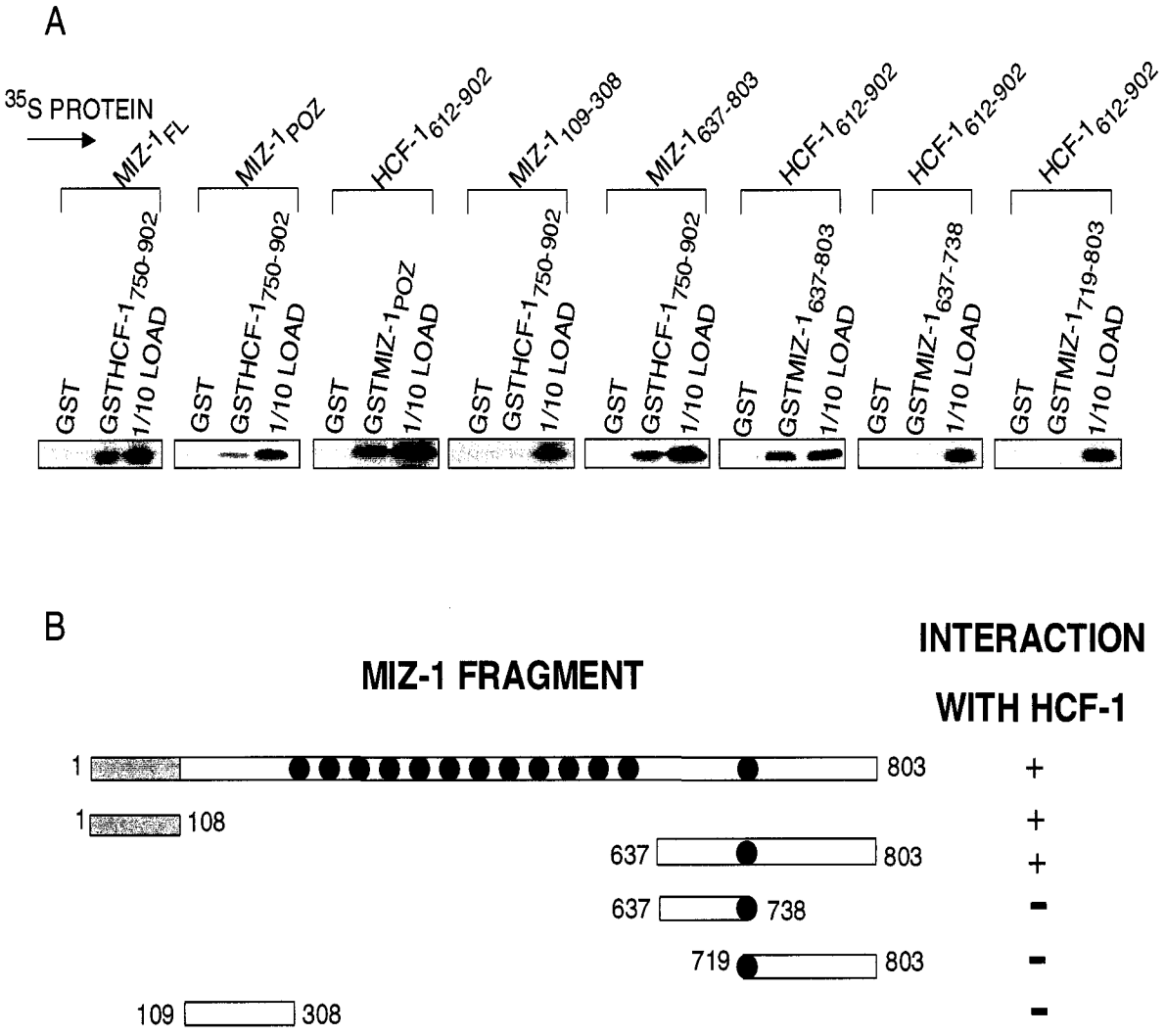


Figure 3.5: HCF-1 interacts directly with Miz-1 *in vitro* and targets two separate domains, the POZ domain and the carboxy-terminal region. A. *In vitro* GST pull-down assay. ^{35}S-methionine labelled Miz-1 or HCF-1 derivatives synthesized *in vitro* were incubated with GST alone or with various GST-fusion proteins as indicated, and bound material was analysed by SDS-polyacrylamide gel electrophoresis. The 1/10 load lanes represent 10% of the ^{35}S-methionine labelled protein added to the respective binding assays. B. Schematic representation of the Miz-1 deletion constructs used above and a summary of the *in vitro* HCF-1-interaction results. +, specific interaction; –, no interaction.

may bind independently to HCF-1. As shown in figure 3.5a, reciprocal GST-binding experiments with Miz-$1_{1\text{-}108}$ demonstrates that the POZ domain itself is sufficient to interact with HCF-1.

Thus, as summarized in figure 3.5b, Miz-1 contains at least two modular sub-domains that can independently bind to HCF-1. These regions are distinct from those that mediate interaction of Miz-1 with c-myc (Peukert *et al.*, 1997), p300 (Staller *et al.*, 2001), SMADs (Seoane *et al.*, 2001), and MAGE-A4 (Sakurai *et al.*, 2004), although both HCF-1 and TopBP1 (Herold *et al.*, 2002) target the Miz-1 POZ domain. The foregoing does not preclude the possibility that subregions of Miz-1 located between residues 108-637, which include the zinc finger cluster, are also important for, or influence, interaction with HCF-1.

3.1.5 HCF-1 targets a transactivation function in Miz-1

Several identified HCF-1 interaction partners, including Miz-1, are transcription factors, consistent with the growing evidence that HCF-1 serves as a global transcriptional co-regulatory factor. Furthermore, recent findings have shown that in some cases HCF-1 is recruited directly to transcriptional activation domains of its interaction partners (Luciano and Wilson, 2000; Vogel and Kristie, 2000a), suggesting that one role of HCF-1 may be to directly modulate the activation potential of its interacting partners.

In order to determine whether the HCF-1 binding interface at the carboxy-terminus of Miz-1 functions as a transactivation domain we sought to identify a transactivation function in Miz-1 and map the localization of its transactivation domain.

To this end, we constructed and tested a series of GAL4-Miz-1 fusion proteins by transient transfection assays in mammalian cells using a luciferase reporter gene driven by a promoter that contained multiple upstream GAL4 binding sites. As shown in figure 3.6, full length Miz-1 (GAL4-Miz-1_{FL}), as well as a derivative lacking the amino-terminal POZ domain (GAL4-Miz-$1_{\Delta POZ}$) resulted in a 13-fold and 31-fold induction of reporter gene activity, respectively, relative to the activity obtained with GAL4 DBD alone. Thus, full length Miz-1, as well as the ΔPOZ derivative, activates transcription when tethered directly to DNA via a heterologous protein.

To determine if the transactivation function mapped to regions required for interaction with HCF-1, we tested the activity of a GAL4 fusion to residues 637-803 of Miz-1. As shown in figure 3.6, GAL4-Miz-$1_{637-803}$ potently activated transcription, resulting in a 253-fold activation in reporter gene expression relative to GAL4 DBD alone. Interestingly, transactivation by this derivative was significantly higher than that observed with the full length protein or the ΔPOZ derivative, a difference that could not be attributed to differences in expression levels of the respective proteins, as determined by Western blot analysis (figure 3.6b, lanes 1, 2, and 4, respectively). This suggests that the more robust activation is an inherent property of this isolated domain, and that the full activation potential of this region may be masked in the context of the intact protein.

Thus, the carboxy-terminal domain of Miz-1 functions as an autonomous transactivation domain in addition to providing an interaction surface for HCF-1. The HCF-1 interaction with Miz-1 was shown in figure 3.5 to require the entire carboxy-terminal domain, indicating that multiple determinants, or an extended region within

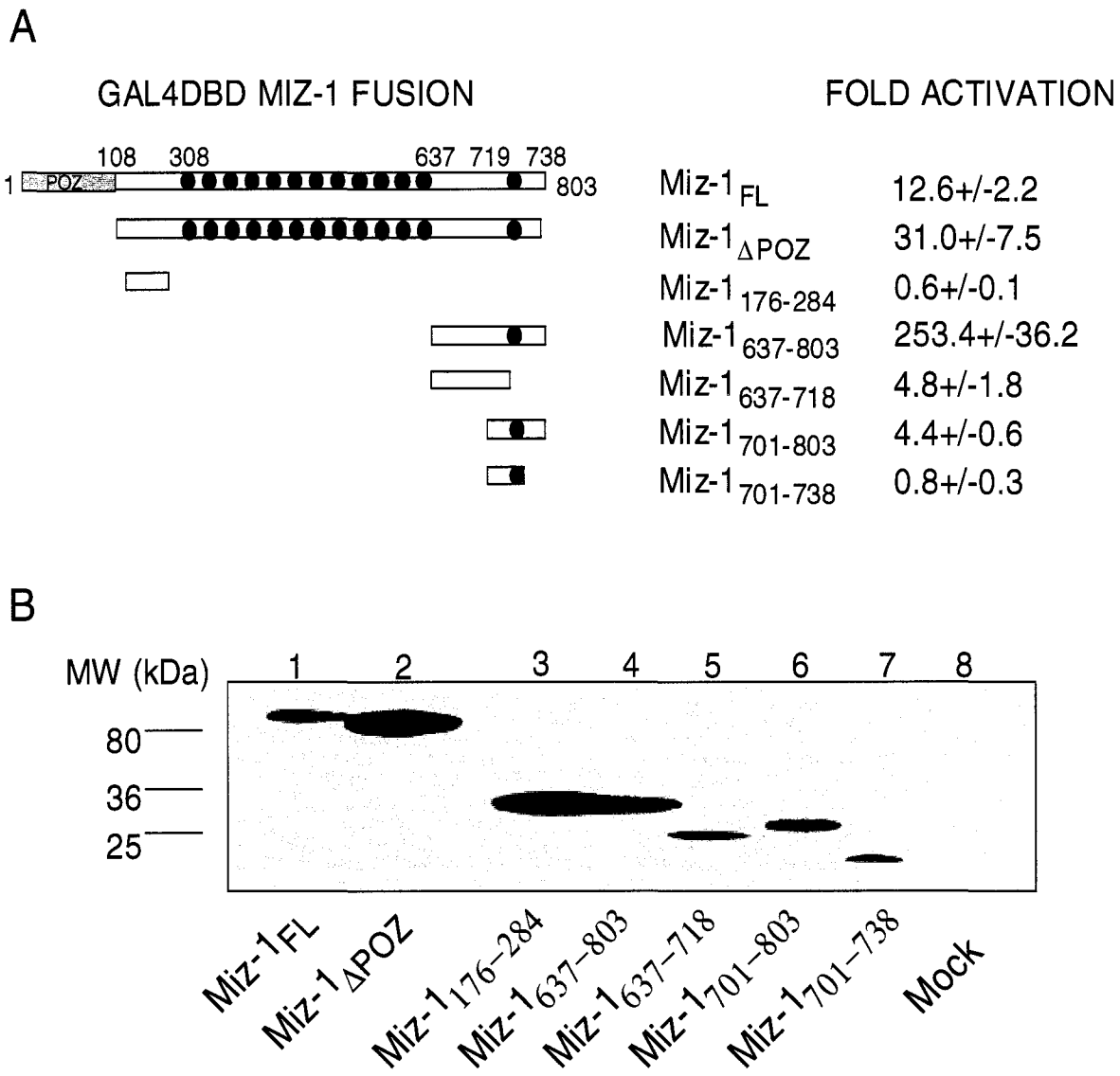


Figure 3.6: The carboxy-terminal HCF-1 interacting interface in Miz-1 functions as an autonomous transactivation domain. A. COS-1 cells were transfected with a GAL4-responsive luciferase reporter plasmid along with expression vectors for various GAL4-Miz-1 fusion derivatives as indicated, and luciferase activity was measured. Values shown represent fold activation relative to GAL4-DBD empty vector control and represent the average activity (+/- SD) from 3 independent transfections carried out in duplicate. B. Expression levels of GAL4DBD-Miz-1 fusion proteins. COS-1 cells were transfected with 1.0 μg of the indicated GAL4-Miz-1 expression plasmid, extracts were resoloved on a SDS-polyacrylamide gel and analyzed by Western blot analysis with mouse monoclonal anti-GAL4DBD antibody. The apparent molecular weight of the products of lanes 3 and 4 appears equal as judged by their migration, although the product of lane 4 should have a greater actual molecular weight. This may be attributable to the high acidic content for the fragment in lane 3 which may have caused it to migrate slower than its actual molecular weight.

residues 637-803 of Miz-1 are required for HCF-1 interaction. In order to determine whether a similar set of determinants were required to form the transactivation domain overlapping subfragments spanning residues 637-803 of Miz-1 were constructed and tested. Overlapping sub-fragments spanning residues 637-718 and 701-803 were marginally active (4-5 fold induction over control), whereas a fragment containing residues 701-738 was inactive. These sub-fragments were also unable to bind to HCF-1. Thus, the Miz-1 transactivation domain may be composed of multiple determinants within residues 637-803. Analysis of the amino acid composition of residues 637-803 reveals that this transactivation domain is not enriched in amino acids often found to compose transactivation domains (Triezenberg, 1995; Kadonaga, 2004). For instance, it is only 14% acidic, 7% glutamine-rich, and 6% proline-rich. However, a moderately charged domain does exist between residues 647-710 where 20% of the residues are acidic and residues 739-803 are enriched for serine and threonine as this region is 16% Ser/Thr. As a control, we also tested residues 176-284, a region which encompasses binding determinants for c-myc (Peukert *et al.*, 1997) and that has been predicted to harbour an activation function because of its high acidic amino acid content (Schneider *et al.*, 1997). However, this region failed to act as an autonomous transactivation domain when fused to the GAL4 DNA binding domain.

These results demonstrate that the carboxy-terminal HCF-1-interacting domain of Miz-1 harbours an autonomous activation function and whose potency correlates with the ability of this region to bind to HCF-1. These findings add to the growing evidence that HCF-1 targets activation domains in its interaction partners.

3.1.6 HCF-1 represses GAL4-Miz-1-mediated transcription activation

Having determined that HCF-1 interacts with the Miz-1 transactivation domain, we sought to elucidate the functional consequences of HCF-1/Miz-1 interaction. We carried out transient transfection assays with various GAL4-Miz-1 fusion proteins in the presence or absence of an expression vector for HCF-1 to determine if HCF-1 modulates transactivation by Miz-1. As shown in figure 3.7a, co-transfection of full length HCF-1 led to a dose-dependent repression of transactivation by GAL4-Miz-1_{FL}. Similarly, HCF-1 inhibited transactivation by a Miz-1 derivative lacking the POZ domain, or a derivative containing only the carboxy-terminal transactivation domain (residues 637-803). In each case, co-transfection of an HCF-1 expression vector led to approximately 70-80% inhibition of activity at the highest concentrations of HCF-1 used. The repressive effect was specific to GAL4-Miz-1 derivatives since similar levels of HCF-1 had no effect on transactivation by GAL4-$VP16_{AAD}$, a GAL4 derivative linked to the potent acidic transactivation domain of VP16. Thus, repression of Miz-1 transactivation is not due to a generalized titration of co-regulatory factors by HCF-1. The observation that HCF-1 represses GAL4-Miz-1_{FL} and GAL4-Miz-$1_{\Delta POZ}$ to a similar degree suggests that inhibition by HCF-1 is independent of the POZ domain. We next tested whether the HCF-1 Miz-1 binding domain (HCF-$1_{750-902}$) could affect GAL4-Miz-1-mediated transactivation. As illustrated in figure 3.7b, HCF-$1_{750-902}$ behaved in a manner analogous to full length HCF-1, inhibiting the transactivation potential of all three GAL4-Miz-1 fusion proteins, but not that of GAL4-$VP16_{AAD}$. Thus, binding of this fragment of HCF-1 directly to the Miz-1 transactivation domain is sufficient to inhibit activity of

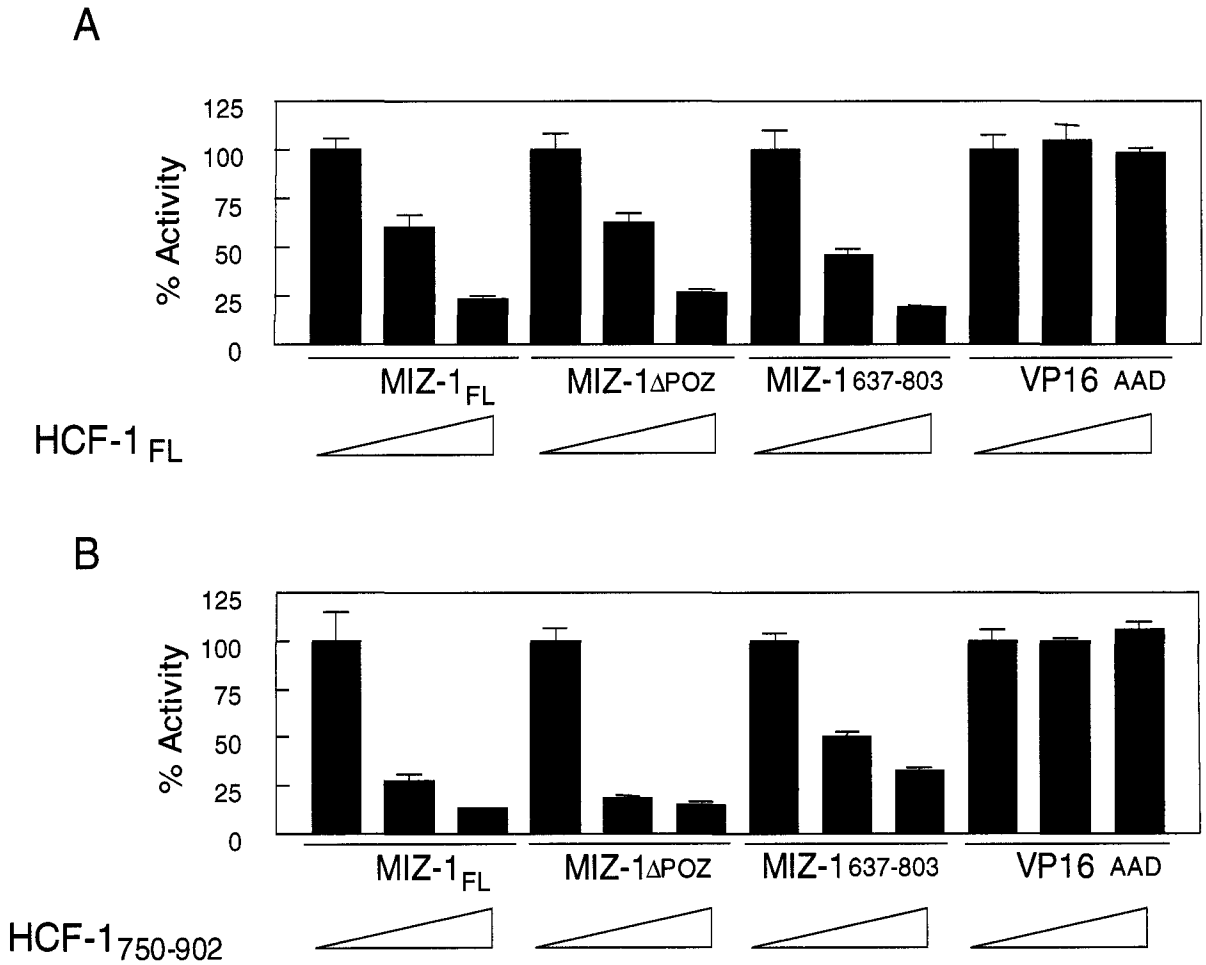


Figure 3.7: HCF-1 antagonizes transactivation by GAL4-Miz-1. A. COS-1 cells were co-transfected with 0.5μg of 5X GAL4 luciferase reporter plasmid, and the indicated GAL4-Miz-1 fusion expression vector (50ng), or GAL4-VP16$_{AAD}$ (50ng) in the absence or presence of increasing amounts of an expression vector for full length HCF-1 (HCF-1$_{FL}$) as indicated. Extracts were prepared 40-48 hours later and assayed for luciferase activity. Values shown represent the average (+/- SD) from 3 independent transfections carried out in duplicate, and normalized to the value obtained from the respective GAL4 fusion effector plasmid alone which was taken as 100% for each case. B. The minimal Miz-1 binding domain of HCF-1 is sufficient to inhibit transactivation by GAL4-Miz-1. Transfections and measurement of luciferase activity was carried out in an identical manner as above except that the expression vector for HCF-1 $_{750\text{-}902}$ was used in place of full length HCF-1.

Miz-1.

3.1.7 HCF-1 represses Miz-1 activation of the $p15^{INK4b}$ promoter

Having shown a repressive effect by HCF-1 on the transcriptional activity of GAL4-Miz-1 fusion proteins, we sought to determine whether a similar effect could be observed with native Miz-1 in the context of a natural Miz-1 target gene. Miz-1 has been shown to stimulate transcription of several cyclin-dependent kinase inhibitors, including $p15^{INK4b}$, through direct binding to the initiator element in these promoters (Staller *et al.*, 2001; Seoane *et al.*, 2002; Adhikary *et al.*, 2003; Wu *et al.*, 2003). To determine if HCF-1 modulates Miz-1-mediated activation of $p15^{INK4b}$ expression, we performed co-transfection assays with a luciferase reporter gene linked to the $p15^{INK4b}$ promoter. As shown in figure 3.8, transfection of an expression vector for full length Miz-1 led to a 7-fold induction of $p15^{INK4b}$ reporter gene activity, consistent with recent findings reported by others (Staller *et al.*, 2001). In contrast, HCF-1 expression had no effect on reporter gene activity. However, co-expression of HCF-1 with Miz-1 inhibited Miz-1 mediated transactivation by approximately 70%. Miz-1 activation of $p15^{INK4b}$ expression is also inhibited by interaction with c-myc (Staller *et al.*, 2001). Consistent with this, transfection of a c-myc expression vector, while having no effect on its own, repressed Miz-1-dependent activation of the reporter gene (see figure 3.8). The extent of repression by c-myc was comparable to that observed with HCF-1. Interestingly, co-expression of both HCF-1 and c-myc led to an additive effect on inhibition of Miz-1 activity, reducing activity down to basal levels observed with the reporter gene alone. Thus HCF-1, like c-myc, antagonizes Miz-1 activation of the native $p15^{INK4b}$ promoter and, furthermore, can

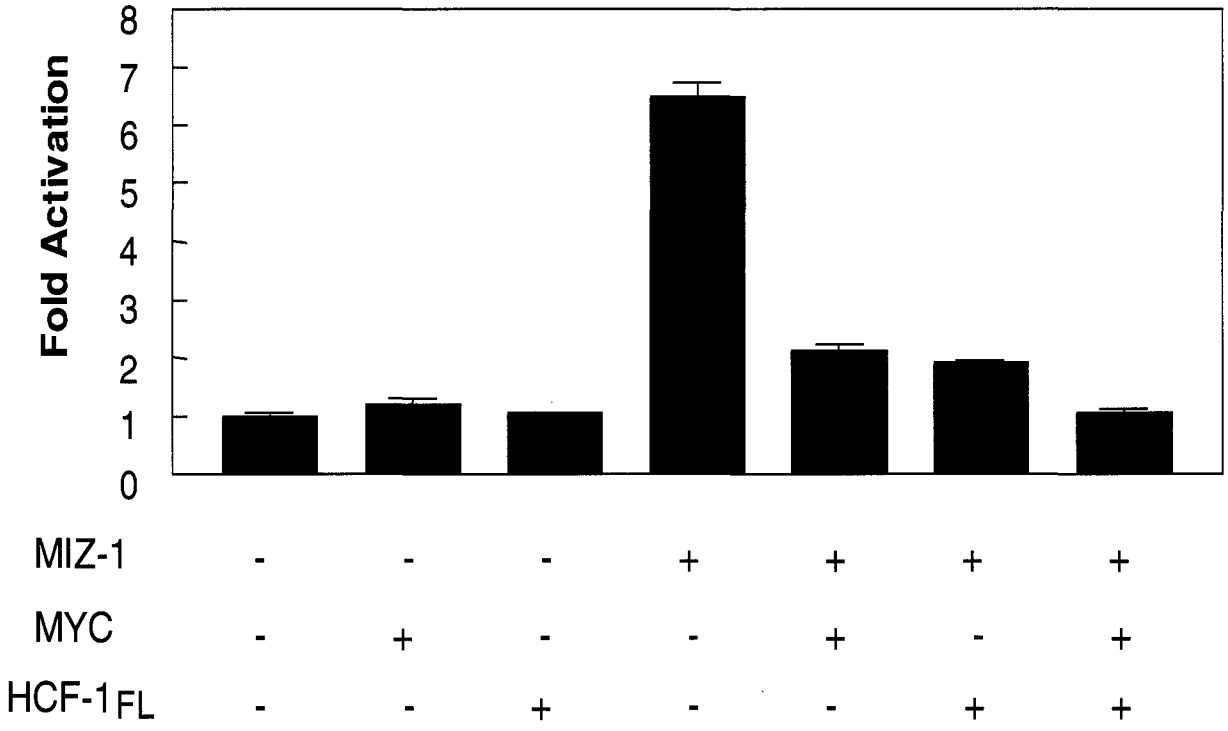


Figure 3.8: HCF-1 represses Miz-1 activation of the p15^{INK4b} promoter. COS-1 cells were co-transfected with a p15^{INK4b} luciferase reporter along with expression plasmids for full length Miz-1, HCF-1, and/or full length c-myc, as indicated. Values represent the average activity (+/- SD) from 3 independent transfections done in duplicate and normalized to the activity obtained from the reporter plasmid alone, which was taken as 1.

cooperate with c-myc in potentiating repression of this target gene.

3.1.8 HCF-1 interferes with recruitment of p300 to Miz-1

C-myc has been shown to inhibit transactivation by Miz-1 by interfering with recruitment of the p300 coactivator (Staller *et al.*, 2001). p300 is a large protein of over 2400 amino acids that contains several independent domains that interact with a large number of cellular proteins in addition to the viral E1A protein (Chan and La Thangue, 2001; Blobel, 2002). p300 has been characterized as a general transcription regulator that enhances transcriptional activation through interaction with the general transcription factors TFIIB, TBP, and RNA polymerase II, thus physically connecting activators with components of the basal transcription machinery (Chan and La Thangue, 2001; Blobel, 2002). In addition, p300 contains a histone acetyltransferase domain, which catalyzes the acetylation of histones, thus influencing chromatin structure and the acetylation of other transcription factors, influencing their activity (Chan and La Thangue, 2001; Blobel, 2002). Due to its function as a general transcription regulator, p300 influences a variety of cellular processes, including cell growth, transformation, and development (Goodman and Smolik, 2000). Because HCF-1 targets a region of Miz-1 that is also involved in p300 association, we speculated that HCF-1 might also function in this manner. To determine whether this is the case, H. Patel carried out a mammalian 2-hybrid assay using GAL4-Miz-1_{FL} and p300-VP16. As shown in figure 3.9a, transfection of cells with the p300-VP16 expression vector on its own had no effect on reporter gene activity. However, co-transfection of expression vectors encoding p300-VP16 and GAL4-Miz-1 led to a 2-fold increase in reporter gene activity compared with cells

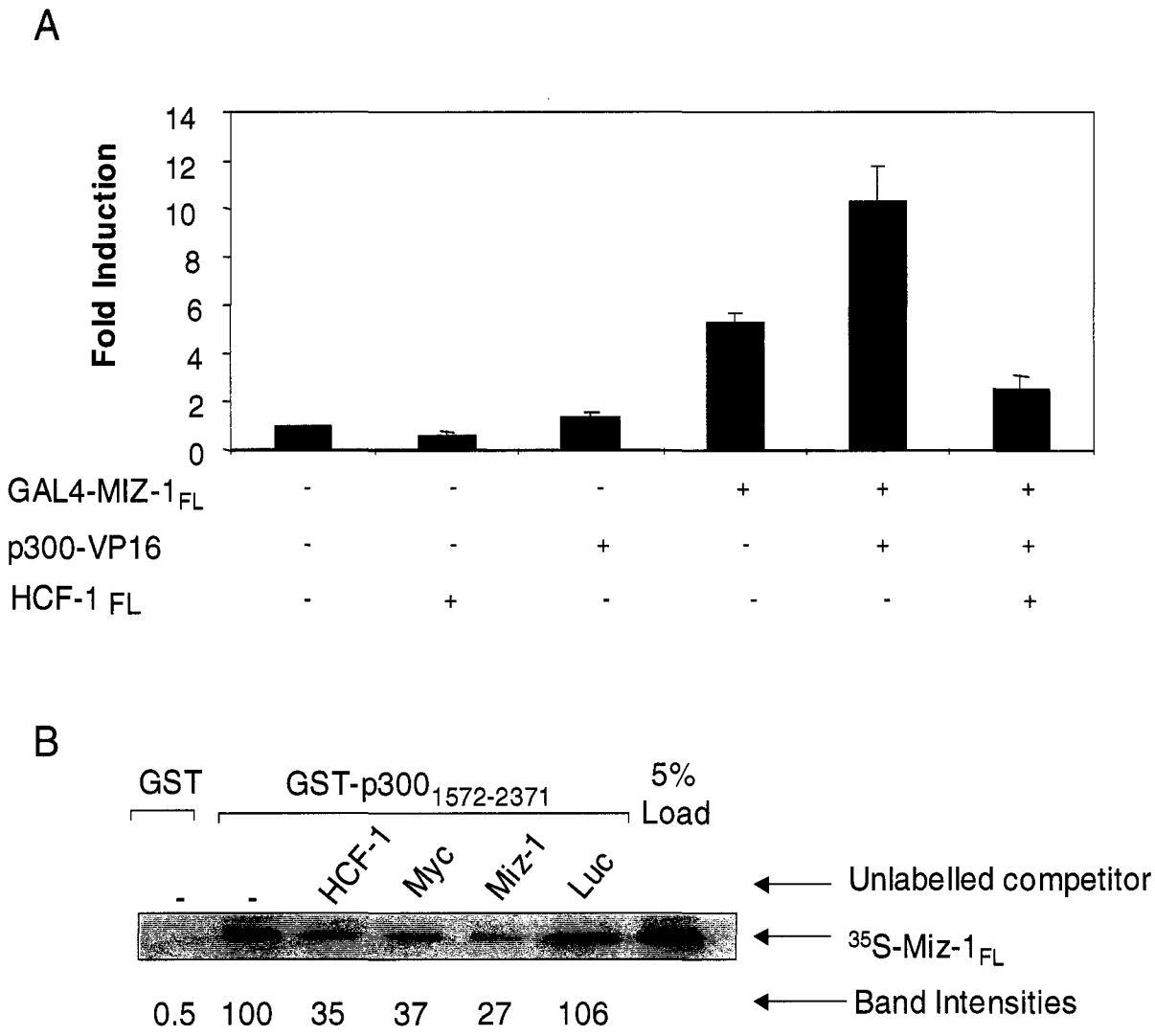


Figure 3.9: HCF-1 inhibits p300 recruitment by Miz-1. A. COS-1 cells were transfected with 0.5μg of a 5X GAL4 luciferase reporter, GAL4-Miz-1_{FL} expression vector (50ng), $p300_{FL}$-VP16 expression vector (0.25μg) and HCF-1_{FL} expression vector (0.25μg) as indicated. Extracts were prepared 40-48 hours later and assayed for luciferase activity. Values shown represent the average (+/-SD) from 2 independent transfections carried out in duplicate. B. *In vitro* GST pull-down assay. ^{35}S-methionine labelled Miz-1_{FL} synthesized *in vitro* was incubated with GST or GST-$p300_{1572-2371}$ as indicated, in the presence or absence of various unlabelled competitor proteins synthesized *in vitro*. Unlabelled competitor proteins included HCF-1_{1-902}, c-myc_{FL}, Miz-1_{FL}, and luciferase. Bound material was analyzed by SDS-polyacrylamide gel electrophoresis. 5% load represents 5% of the ^{35}S-methionine labelled protein added to the binding assays. Band intensities were determined by PhosphorImager analysis. Band intensity was set at 100 for the reaction lacking unlabelled competitor and other bands were scored relative to this. Panel A of this figure represents the work of H. Patel.

transfected with GAL4-Miz-1 alone, similar to what has been previously observed (Staller *et al.*, 2001). Inclusion of an expression vector encoding full length HCF-1 abrogated the stimulatory effect of p300-VP16 and further repressed GAL4-Miz-1 transactivation potential. To determine whether HCF-1 was interfering with the recruitment of p300 to Miz-1, we used the GST pull-down competition assay with a carboxy-terminal fragment of p300 (residues 1572-2371) that has been shown to interact with Miz-1 (Staller *et al.*, 2001) fused to GST along with radiolabelled full length Miz-1 and unlabeled competitor proteins synthesized *in vitro*. As shown in figure 3.9b, full length Miz-1 bound to GST-$p300_{1572-2371}$ as expected. Inclusion of equivalent amounts of unlabeled HCF-1_{1-902}, c-myc, or Miz-1 (as a positive control) reduced binding of radiolabeled Miz-1 by 60-70%. In contrast, inclusion of unlabeled luciferase (negative control) had no effect on binding. Thus, HCF-1 competes with p300 for interaction with Miz-1.

3.1.9 The P134S mutation in HCF-1 does not affect binding to, or transactivation by, Miz-1

An HCF-1 N-terminal fragment spanning residues 1-902, which includes the basic domain that is targeted by Miz-1, is necessary and sufficient to rescue temperature-sensitive cell cycle arrest in tsBN67 cells (Wilson *et al.*, 1997). The causative mutation in HCF-1 (P134S), in addition to preventing chromatin association (Wysocka *et al.*, 2001), pre-mRNA processing (Ajuh *et al.*, 2002), and VP16 association (Wilson *et al.*, 1997), also abrogates interaction with cellular proteins such as Luman/LZIP (Freiman and Herr, 1997), although Luman/LZIP interaction is incidental to the ability of HCF-1 to promote cell cycle progression (Mahajan and Wilson, 2000). We wished to determine

whether the point mutation in HCF-1 that causes cell cycle arrest also abrogates Miz-1 binding and/or the ability of HCF-1 to influence Miz-1 activity. Although the N-terminal HCF-1 kelch domain, which harbours proline 134, is not necessary for Miz-1 interaction, the possibility remains that in the context of the native protein or larger sub-fragments of HCF-1, the P134S point mutation may induce long range perturbations that could modulate Miz-1 binding. To test this directly, we used GST pull-down assays with GST-Miz-$1_{637\text{-}803}$ and *in vitro* synthesized, radiolabelled wild type HCF-$1_{1\text{-}902}$ or the corresponding HCF-1 fragment harbouring the P134S point mutation. As expected, radiolabelled $HCF_{1\text{-}902}$ bound to GST-Miz-$1_{637\text{-}803}$ but not to GST alone (figure 3.10a). In addition, HCF-$1_{1\text{-}902P134S}$ bound to GST-Miz-$1_{637\text{-}803}$ as efficiently as the wild type HCF-1. To ensure the specificity and selectivity of the assay, we tested HCF-1 binding to GST-$VP16_{1\text{-}404}$, a GST fusion protein linked to residues 1-404 of VP16 which contains determinants necessary for interaction with HCF-1 (Popova *et al.*, 1995). As expected, $HCF_{1\text{-}902}$ but not HCF-$1_{1\text{-}902P134S}$ bound to GST-$VP16_{1\text{-}404}$. Although direct interaction between HCF-1 and Miz-1 is unaffected by the P134S mutation in HCF-1, the possibility remained that the HCF-1 mutation may affect the ability of HCF-1 to influence Miz-1 activity. To determine whether the P134S mutation affects the ability of HCF-1 to attenuate Miz-1-mediated transactivation, we carried out transient transfection experiments similar to those described for figure 3.7. As shown in figure 3.10b, full length HCF-1 harbouring the P134S mutation retained the ability to inhibit transactivation by Miz-1. Thus, under these experimental conditions, the causative point mutation in HCF-1 that is responsible for the temperature-sensitive cell cycle arrest in

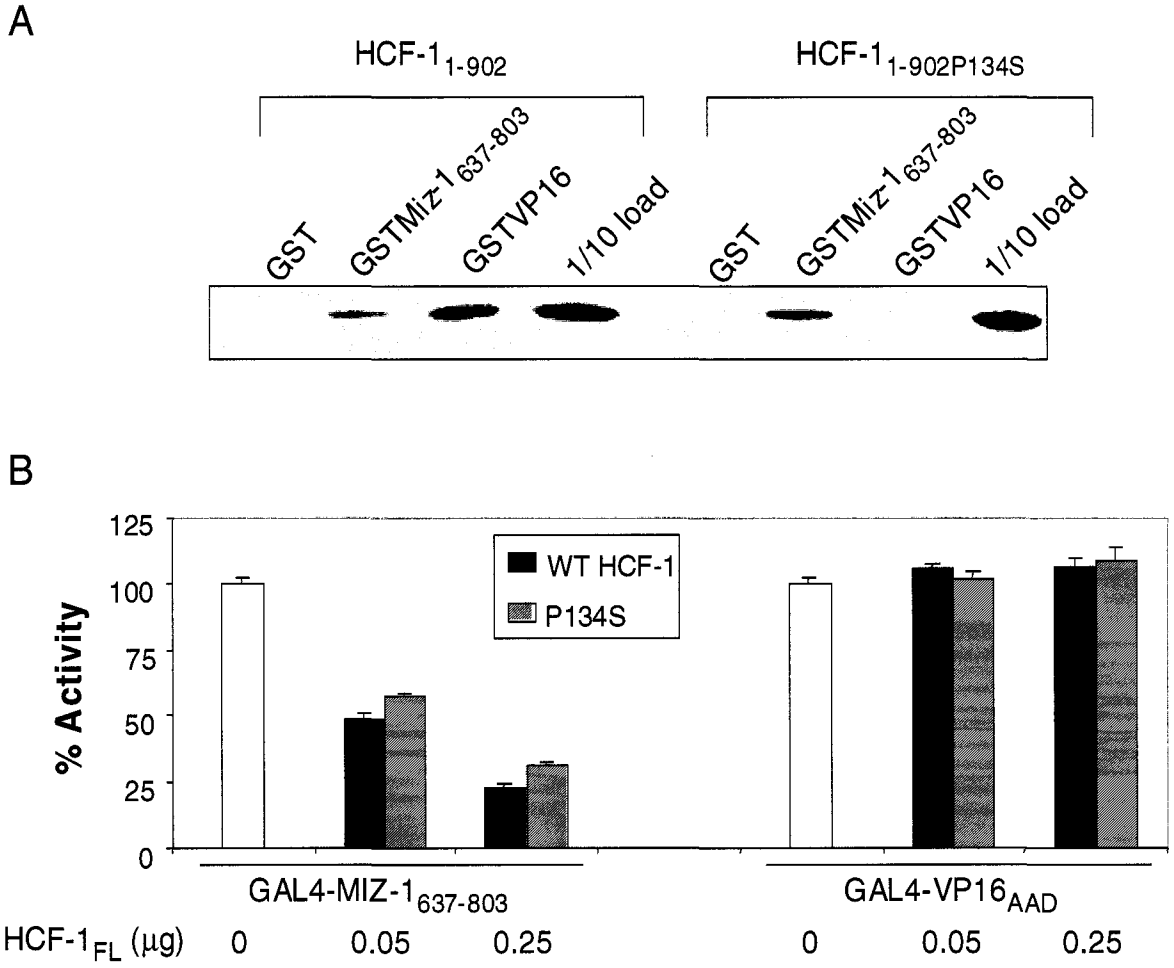


Figure 3.10: The P134S mutation in HCF-1 does not inhibit binding to, or transactivation by, Miz-1. A. *In vitro* synthesized ^{35}S-methionine-labeled HCF-1(1-902) or HCF-1(1-902)(P134S) was incubated with GST, GST-Miz-1(637-803), or GST-VP16(1-404) as indicated, and bound material was analyzed by SDS-PAGE. 1/10 load represents 10% of the ^{35}S-methionine-labeled protein used in the respective binding assays. B. COS-1 cells were co-transfected with a GAL4-responsive luciferase reporter plasmid and the indicated GAL4-Miz-1 fusion expression vector or GAL4-VP16AAD in the absence or presence of increasing amounts of an expression vector for full length (wild type (WT)) HCF-1 or HCF-1 (P134S) as indicated and assayed for luciferase activity as described in the legend to figure 3.7.

tsBN67 cells does not interfere with the ability of HCF-1 to associate with Miz-1 or inhibit transactivation by Miz-1.

3.2 INVESTIGATING THE IMPORTANCE OF MIZ-1 AND OTHER HCF-1 BASIC DOMAIN INTERACTION PARTNERS IN tsBN67 PROLIFERATION

3.2.1 Identification of an HCF-1 mutant compromised in its ability to bind GABP

The basic domain of HCF-1 has been shown to be necessary for cellular proliferation of tsBN67 cells (Wilson *et al.*, 1997). However, this region has been shown to interact with a variety of cellular proteins, in addition to Miz-1 (Gunther *et al.*, 2000; Vogel and Kristie, 2000a; Wysocka *et al.*, 2003). How any of the basic domain interacting proteins contribute to HCF-1-mediated cellular proliferation is not currently known. We intended to determine whether the interaction with Miz-1 is necessary for HCF-1-mediated complementation of the temperature-sensitive cellular proliferation defect in tsBN67 cells by constructing HCF-1 mutants impaired in their ability to bind Miz-1. In order to construct a manageable number of HCF-1 mutants, we first sought to further localize a region of HCF-1 that mediated interaction with Miz-1. We had previously used the yeast 2-hybrid interaction assay to determine that residues 750-902 in HCF-1 were sufficient for this function (see figure 3.4), and thus created further N- and C-terminal deletions within GAL4-DBD-HCF-$1_{750\text{-}902}$ and tested these for interaction by yeast 2-hybrid analysis with GAL4-AD-Miz-1 to minimize the number of residues we would have to mutate (see figure 3.11). These experiments demonstrate that HCF-1 residues 770-806 are sufficient to interact with Miz-1.

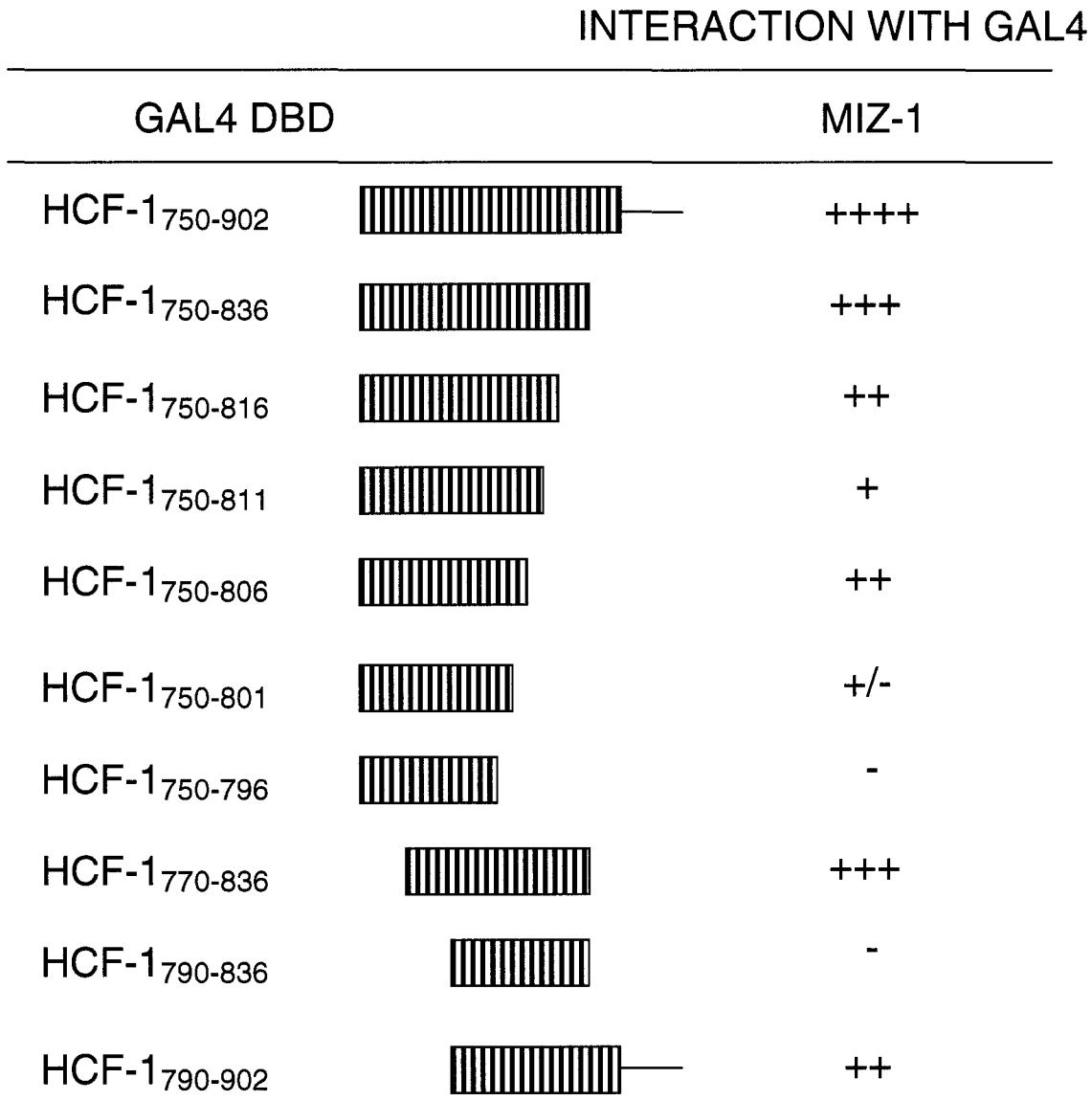


Figure 3.11: Identification of a small region in HCF-1 that mediates binding to Miz-1. Yeast 2-hybrid assay was utilized to detect interaction between HCF-1 constructs and Miz-1. Yeast strain, Y190, was transformed with the indicated plasmids and grown on appropriate media for 3-4 days. β-gal activity was determined qualitatively using β-gal overlay assay with X-gal as substrate. Each plus sign represents 25% of full activity (or 25% of the strongest interaction) as estimated from the intensity of blue.

With this information we next constructed a series of triple alanine mutations or internal deletions within a fragment of HCF-1 encompassing residues 750-902 fused to the GAL4-DBD. The mutants were tested for interaction with GAL4-AD fusions to Miz-1 in a quantitative yeast 2-hybrid assay, as shown in figure 3.12. However, a mutant displaying a reduced ability to bind Miz-1 was not found. We believe that this is the result of the nature of the HCF-1/Miz-1 interaction. Miz-1 may contact several clusters of amino acids over a region of HCF-1 spanning residues 770-902, and an analysis of the data from figure 3.11 reveals that although regions 770-790, 796-806, and 836-902 of HCF-1 are necessary for Miz-1 interaction when assayed within smaller fragments of the HCF-1 basic domain lacking compensatory regions, these regions are not necessary to interact with Miz-1, when only one region is mutated or deleted in the context of the entire basic domain. Thus, mutation of one cluster of amino acids in HCF-1 is insufficient to abrogate binding to Miz-1. Our inability to generate an HCF-1 mutant impaired in its ability to interact with Miz-1 is reminiscent of similar problems reported by others in generating a pRB mutant impaired in its interaction with E2F factors (Dick and Dyson, 2002). Similar to the situation with HCF-1/Miz-1, the crystal structure of an 18 amino acid peptide from the carboxy-terminus of E2F with the small pocket of pRB revealed that several clusters of amino acids in the pRB pocket directly contacted the E2F peptide (Dick and Dyson, 2003). Consequently, mutation of any one of these clusters alone was insufficient to disrupt the pRB/E2F interaction (Dick and Dyson, 2002). Indeed, simultaneous substitution of four clusters of amino acids within the pocket was necessary to disrupt binding (Dick and Dyson, 2002). Thus, the nature of the HCF-

	INTERACTION (β-GAL VALUES)	
GAL4 DBD HCF-1 CONSTRUCT	MIZ-1	GABP
WILD TYPE	41.2+/-2.8	1.6+/-0.06
769-771Ala	44.4+/-2.7	1.0+/-0.07
772-774Ala	46.2+/-1.3	1.8+/-0.08
775-777Ala	49.1+/-3.9	1.8+/-0.06
778-780Ala	54.6+/-4.9	2.1+/-0.13
781-783Ala	38.6+/-3.8	1.2+/-0.04
784-786Ala	48.5+/-3.1	2.2+/-0.06
786-787Ala	44.0+/-4.7	2.2+/-0.07
788-789Ala	44.0+/-2.6	2.3+/-0.2
790-792Ala	48.2+/-3.8	2.1+/-0.09
793-795Ala	55.4+/-1.6	0.6+/-0.07
796-798Ala	41.7+/-1.6	2.2+/-0.05
799-801Ala	50.3+/-2.7	2. 4+/-0.1
Δ797-801	47.9+/-2.3	3.2+/-0.2
Δ802-806	59.2+/-5.2	3.0+/-0.4
Δ797-806	44.6+/-4.0	2.9+/-0.2

Figure 3.12: HCF-1 mutant 793-795Ala is impaired in its interaction with GABP but not Miz-1. Yeast 2-hybrid assay was utilized to detect interaction between HCF-1 mutants and Miz-1 or GABP. Yeast strain Y190 was transformed with the indicated plasmids and grown for 3-4 days on appropriate media. Interaction was detected using quantitative β-galactosidase assay using ONPG as substrate. The values shown represent the average ß-galactosidase activity (+/- SD) from three independent transformants assayed in duplicate. Mutations were made by PCR mutagenesis (Stratagene QuikChange Mutagenesis) using HCF-1 (750-902) as the wild type. Nomenclature is as follows: "769-771Ala" indicates that residues 769, 770, and 771 have been changed to alanine within the HCF-1 (750-902) background. Δ797-801 indicates that residues 797-801 inclusive have been deleted from the HCF-1 (750-902) background.

1/Miz-1 interaction may be similar to the nature of the pRB/E2F interaction, and thus without prior knowledge of the three dimensional structure of the HCF-1/Miz-1 interaction, generation of an HCF-1 mutant impaired in its ability to interact with Miz-1 will prove equally difficult to attain.

Although unable to isolate a mutation that reduced binding to Miz-1, we also tested whether any of the mutations we had created in HCF-1 could influence binding to GABP, another HCF-1 binding partner which also targets the basic domain (Vogel and Kristie, 2000a). In fact, a mutant in which amino acids 793-795 were mutated to alanine exhibited nearly three fold less binding to GABP, whereas binding to Miz-1 was slightly increased, indicating that this mutant fragment of HCF-1 is folding into a functional polypeptide. Therefore, although we have not isolated an HCF-1 mutant compromised in its ability to bind Miz-1, we have found one compromised in its ability to bind GABP. In addition to the 793-795Ala mutant, two other triple alanine mutations, 769-771Ala and 781-783Ala, were shown to have a somewhat reduced capability to bind GABP, although not to the extent of the 793-795Ala mutant (see figure 3.12).

3.2.2 HCF-1 793-795Ala mutant is also compromised in its ability to rescue the tsBN67 cell cycle defect

Having identified an HCF-1 mutant compromised in its ability to bind GABP, we next exploited this finding to determine the importance of GABP in HCF-1-mediated cell cycle progression by testing the ability of this mutant to complement the tsBN67 cell cycle proliferation defect. We thus conducted a commonly used assay for HCF-1 function in which the ability of HCF-1 to rescue cell proliferation in a temperature-

sensitive cell line, tsBN67$_{HR1}$, was assessed (Wilson *et al.*, 1997; Scarr and Sharp, 2002). tsBN67$_{HR1}$ cells were transfected with pSV2-NEO along with one of the following; HCF-1$_{1-1011}$, HCF-1$_{1-1011}$P134S, HCF-1$_{1-1011}$769-771Ala, HCF-1$_{1-1011}$781-783Ala, or HCF-1$_{1-1011}$793-795Ala as shown in figure 3.13a. Transfected cells were split two days later in media containing G418, to eliminate untransfected cells, and grown at 33.5°C or 40°C for three weeks and visualized by crystal violet staining. Growth at 33.5°C was relatively equivalent in all transfections (figure 3.13a; compare plates a, c, e, g, i, k) indicating equivalent transfection efficiencies. As expected, the P134S mutation served as a negative control, and failed to complement the temperature-sensitive defect (compare plates b, d, and f). The 769-771Ala and 781-783Ala mutants which bound GABP to nearly wild type levels, as shown in figure 3.12, rescued the temperature-sensitive defect to nearly wild type levels although they were somewhat compromised in their ability to complement the temperature-sensitive defect compared to wild type HCF-1$_{1-1011}$ (compare plates d, h, and j). On the other hand, the 793-795Ala mutant which had a 3-fold less ability to bind HCF-1 was also compromised in its ability to complement the tsBN67 defect as considerably fewer colonies were evident on its plate as compared to the wild type HCF-1$_{1-1011}$ plate (compare plates d and l). A Western blot, shown in figure 3.13b, demonstrates that wild type HCF-1$_{1-1011}$ and the novel mutants were expressed to comparable levels in tsBN67$_{HR1}$ cells following transfection. Thus, these results indicate that an interaction with GABP is required for HCF-1-mediated rescue of the temperature-sensitive tsBN67 cellular proliferation defect.

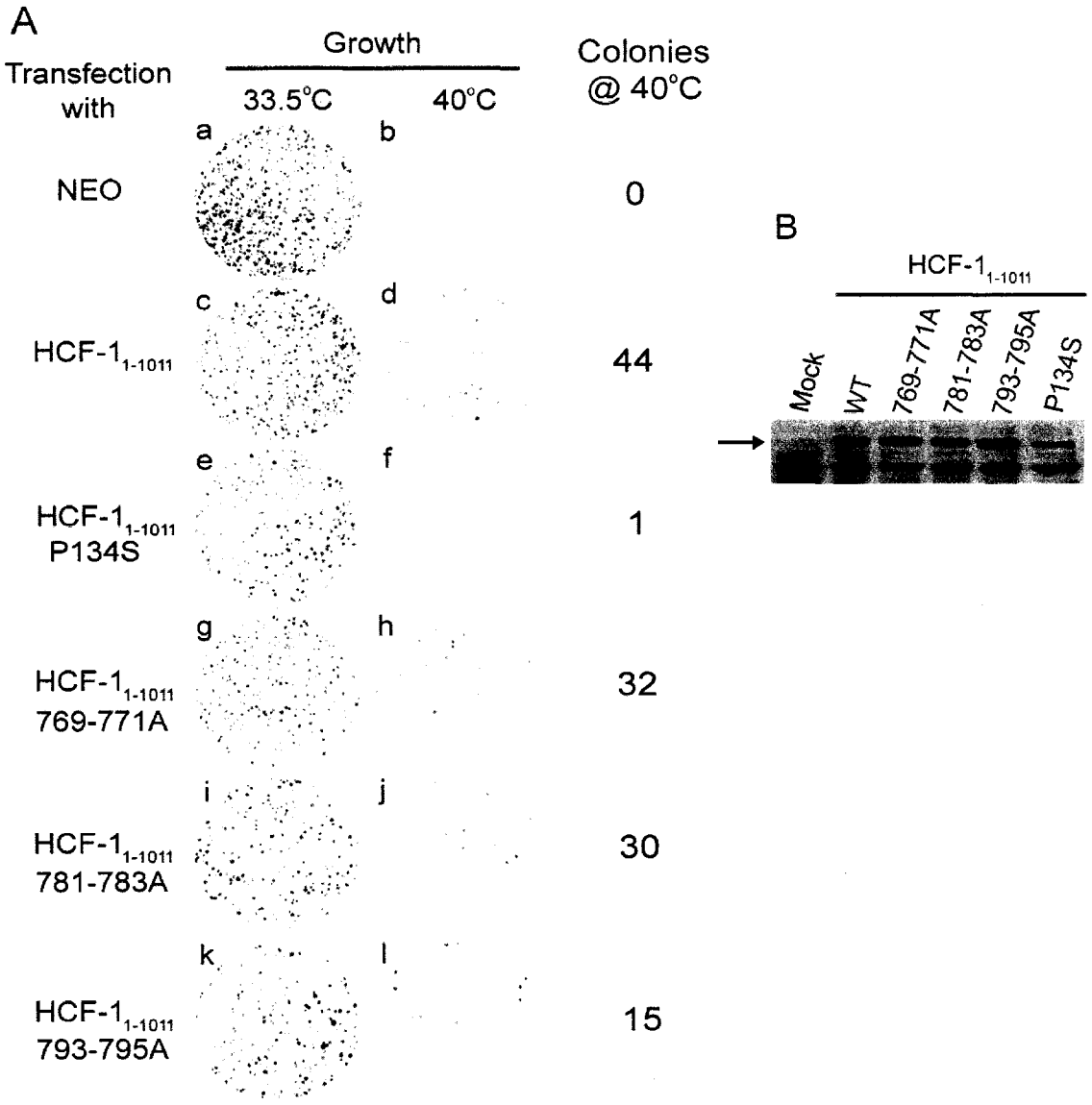


Figure 3.13: HCF-1 mutant 793-795Ala, impaired in its interaction with GABP, reduces rescue of the tsBN67 temperature-sensitive cellular proliferation defect. A. tsBN67$_{HR1}$ cells were transfected with pSV2-NEO, HCF-1$_{1-1011}$, or mutants of HCF-1 as shown. Cells were grown at 33.5°C or 40°C under G418 selection for 3 weeks and visualized by staining with crystal violet. Colonies @ 40°C represents the number of colonies visible on the plate following 3 weeks of growth from the representative experiment. B. The expression levels of HA-tagged HCF-1 constructs were determined by Western blotting with anti-HA antibody. The arrow indicates the position of the HCF-1 constructs. Lysates were produced from tsBN67$_{HR1}$ cells transfected with 1.0μg of the expression vector for the indicated HCF-1$_{1-1011}$ constructs. The results show representative plates from an experiment performed three times.

3.2.3 Miz-1 does not prevent HCF-1-mediated rescue of the temperature-sensitive cellular proliferation defect in tsBN67$_{HR1}$ cells

The necessity of Miz-1 in tsBN67 cellular proliferation could not be elucidated since we did not selectively disrupt Miz-1 binding to HCF-1. However, we sought additional assays to determine whether Miz-1 was of importance in this process. Having demonstrated that HCF-1 interacts with Miz-1 and reduces the ability of Miz-1 to upregulate the expression of p15^{INK4b}, a cyclin-dependent kinase inhibitor, we asked whether overexpression of Miz-1 would affect the ability of HCF-1 to rescue the proliferation defect in tsBN67$_{HR1}$ cells. Miz-1 has been shown to upregulate the expression of p15^{INK4b} (Seoane *et al.*, 2001; Staller *et al.*, 2001), p21^{Cip1} (Seoane *et al.*, 2002; Wu *et al.*, 2003), and p57^{Kip2} (Adhikary *et al.*, 2003), cyclin-dependent kinase inhibitors, and result in cell cycle arrest at G1 in a variety of cell lines (Peukert *et al.*, 1997; Staller *et al.*, 2001; Herold *et al.*, 2002; Zhao *et al.*, 2004). Interestingly, this effect requires the Miz-1 DNA binding domain, indicating that transcriptional control is involved in this mechanism (Peukert *et al.*, 1997). Furthermore, a Miz-1 construct lacking the POZ domain is compromised in its ability to activate transcription and promote cell cycle arrest (Herold *et al.*, 2002). Thus, whereas overexpression of full length Miz-1 results in cell cycle arrest in a variety of cell lines, overexpression of Miz-1$_{\Delta POZ}$ does not (Herold *et al.*, 2002). Together, these results suggest that Miz-1-induced cell cycle arrest occurs by transactivation of Miz-1 target genes, and that the Miz-1 POZ domain is required for transcriptional activation and growth suppression by Miz-1. We therefore wished to determine whether overexpression of Miz-1$_{FL}$ would override HCF-1-mediated cell cycle progression in tsBN67$_{HR1}$ cells and whether deleting the POZ domain

from Miz-1 would compromise this function. tsBN67$_{HR1}$ cells were transfected with pSV2-NEO, or HCF-1$_{1-1011}$, in combination with wild type Miz-1, Miz-1$_{\Delta POZ}$, or the Miz-1 empty vector as shown in figure 3.14. Growth at 33.5°C was relatively equivalent in all transfections (figure 3.14; compare plates a, c, e, g) indicating equivalent transfection efficiencies. As expected, HCF-1 overexpression gave rise to an increase in colony formation at the non-permissive temperature (compare plates b and d), indicating a rescue of temperature-sensitive arrest as shown previously (Wilson et al., 1997; Reilly and Herr, 2002; Scarr and Sharp, 2002). However, unlike previously reported findings in which overexpression of Miz-1 caused arrest of cellular proliferation in a variety of cell lines (Peukert *et al.*, 1997; Staller *et al.*, 2001; Herold *et al.*, 2002), co-transfection of Miz-1$_{FL}$ in conjunction with HCF-1$_{1-1011}$ did not prevent HCF-1-mediated rescue. Miz-1$_{\Delta POZ}$ acted similarly to full length Miz-1 in this assay, in that it did not prevent rescue by HCF-1$_{1-1011}$. These results indicate that Miz-1 cannot overcome an HCF-1-mediated cellular proliferation rescue of tsBN67$_{HR1}$ cells at the non-permissive temperature. In fact, the results presented here suggest that the presence of Miz-1 may augment rescue of tsBN67$_{HR1}$ cells in conjunction with HCF-1. This seems paradoxical since Miz-1 is characterized as a cell cycle inhibitory factor that contributes to cell cycle arrest following DNA damage and differentiation signals (Seoane *et al.*, 2001; Herold *et al.*, 2002; Seoane *et al.*, 2002; Wu *et al.*, 2003; Zhao *et al.*, 2004). However, in contrast to studies where Miz-1 overexpression led to growth arrest, more recent results using siRNAs to ablate Miz-1 expression in HepG2 cells indicate that Miz-1 may be required for cellular proliferation (Ziegelbauer *et al.*, 2004). Thus, the function of Miz-1 may be

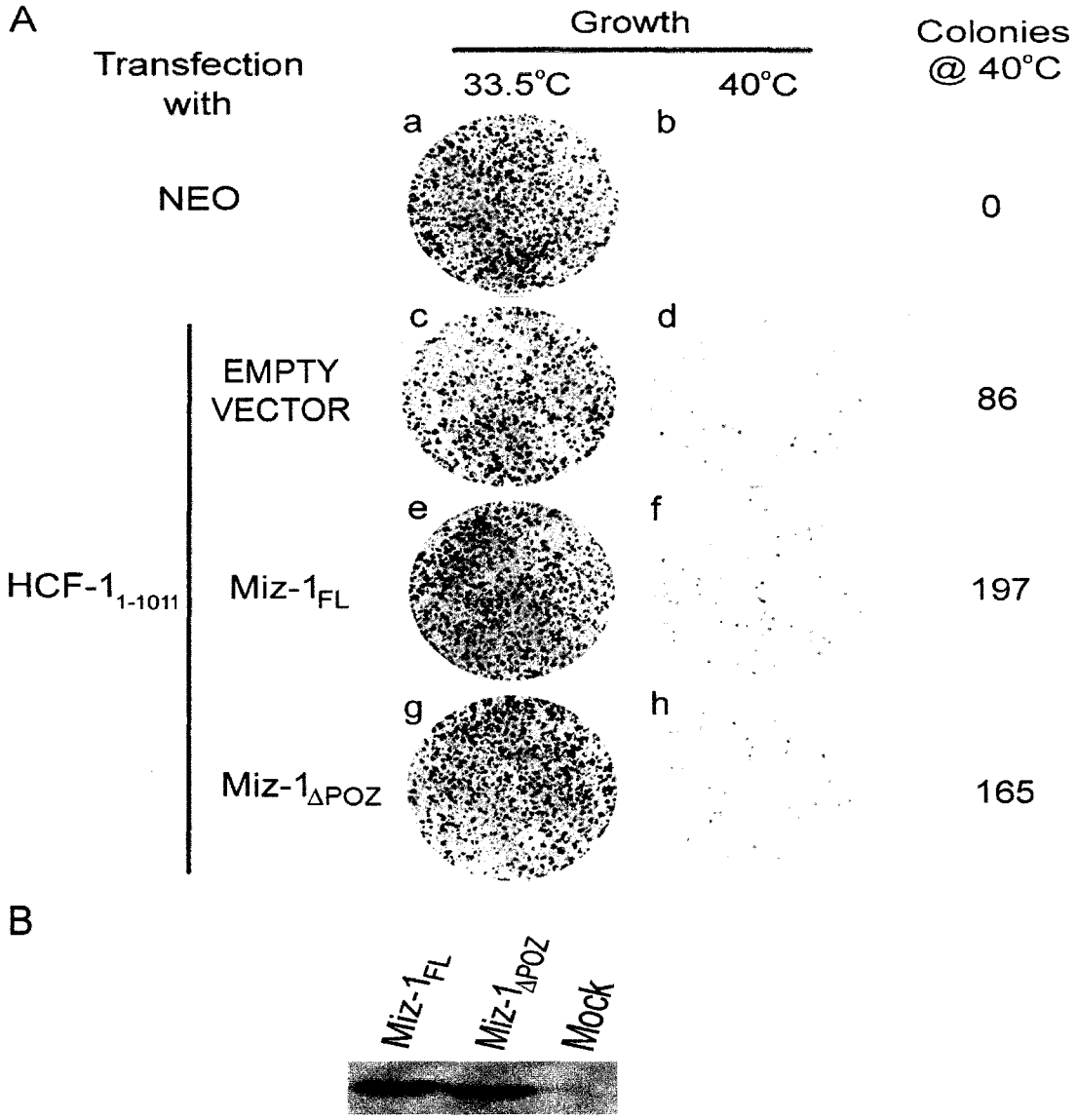


Figure 3.14: Miz-1 does not prevent HCF-1-mediated rescue of the temperature-sensitive cellular proliferation defect in tsBN67$_{HR1}$ cells. A. tsBN67$_{HR1}$ cells were transfected with pSV2-NEO, or HCF-1$_{1-1011}$, in conjunction with wild type V5-Miz-1, V5-Miz-1$_{\Delta POZ}$, or empty vector, as shown. Cells were grown at 33.5°C or 40°C under G418 selection for 3 weeks and visualized by staining with crystal violet. Colonies @ 40°C represents the number of colonies visible on the plate following 3 weeks of growth from the representative experiment. B. The expression levels of wild type V5-Miz-1 and V5-Miz-1$_{\Delta POZ}$ were determined by Western blotting with anti-V5 antibody. Lysates were produced from tsBN67$_{HR1}$ cells transfected with 1.0μg of the expression vector for the indicated Miz-1 construct. The results show representative plates from an experiment performed three times.

cell type specific where its expression level or presence of interaction partners determines whether it results in cell cycle arrest or cellular proliferation. The results presented here suggest that when overexpressed in tsBN67$_{HR1}$ cells, Miz-1 may promote cellular proliferation in conjunction with HCF-1. A Western blot is shown in figure 3.14b demonstrating that both Miz-1$_{FL}$ and Miz-1$_{\Delta POZ}$ were expressed to comparable levels in tsBN67$_{HR1}$ cells following transfection.

3.2.4 Fragments of HCF-1 that selectively interact with Miz-1 do not affect rescue of tsBN67$_{HR1}$ cells by HCF-1$_{1-1011}$

Previous work by Sharp and co-workers has demonstrated an interaction between HCF-1 and PDCD2 (Scarr and Sharp, 2002), the human homologue of a rat protein associated with apoptosis (Kawakami *et al.*, 1995). They have demonstrated that overexpression of PDCD2 overcomes HCF-1-mediated rescue of the tsBN67$_{HR1}$ cellular proliferation defect at the non-permissive temperature and that fragments of HCF-1 that selectively bind PDCD2 augment the rescue by HCF-1, presumably by binding to endogenous PDCD2 and preventing their negative effects on HCF-1 (Scarr and Sharp, 2002). Although, we had been unable to demonstrate an inhibitory effect by Miz-1 on HCF-1-mediated rescue of tsBN67$_{HR1}$ cells, we wished to determine whether fragments of HCF-1 that selectively interact with Miz-1, could influence rescue by co-transfected HCF-1$_{1-1011}$. In order to test this possibility, we first isolated fragments of HCF-1 that selectively bound Miz-1. The HCF-1 basic domain, at the time of these experiments, had been shown to interact with two other cellular transcription factors, GABP (Vogel and Kristie, 2000a) and Sp1 (Gunther *et al.*, 2000). Although the Sp1 interaction site is entirely downstream of the Miz-1 binding site and thus functionally distinct (Gunther *et*

al., 2000), the GABP interaction site (amino acids 813-847 of HCF-1) (Vogel and Kristie, 2000a) overlaps with the Miz-1 interaction site (amino acids 750-902 of HCF-1). Thus, we further delineated these interaction sites in order to uncover a fragment that selectively bound Miz-1. To accomplish this we utilized the series of deletions within GAL4-DBD-HCF-$1_{750\text{-}902}$ we had constructed previously (see figure 3.11) and tested these for interaction by yeast 2-hybrid analysis with both GAL4-AD-Miz-1 and –GABP. As shown in figure 3.15a, although Miz-1 and GABP both bound to HCF-1 residues 750-902 as expected, they exhibited different requirements as illustrated by a number of key deletions. Most importantly, although HCF-1 residues 770-836 and 790-902 were sufficient to bind Miz-1, residues 790-836 were not, indicating that two regions of HCF-1 within residues 770-902 are important for Miz-1 interaction. Specifically, these results indicate that a region encompassed by residues 770-790, and a separate region encompassing HCF-1 residues 836-902 are both involved in Miz-1 interaction. Conversely, interaction between HCF-1 and GABP was much more sensitive to several of the HCF-1 deletions. The results with GABP are consistent with reports by Vogel and Kristie that HCF-1 residues 813-847 are critical to maintain an HCF-1/GABP interaction (Vogel and Kristie, 2000a). Thus, our results demonstrate that deleting residues 816-836 severely impairs the HCF-1/GABP interaction; allowing us to distinguish regions of the HCF-1 basic domain that contribute to the distinct interactions with Miz-1 and GABP. More importantly, these experiments have uncovered a region (residues 770-836) of HCF-1 that selectively maintains interaction with Miz-1. In order to confirm that a fragment of HCF-1 spanning residues 770-836 was sufficient to interact with Miz-1, we

A

GAL4 DBD	MIZ-1	GABP
	INTERACTION WITH GAL4 AD	
$HCF\text{-}1_{750\text{-}902}$	++++	+++
$HCF\text{-}1_{750\text{-}836}$	+++	+
$HCF\text{-}1_{750\text{-}816}$	++	+/-
$HCF\text{-}1_{750\text{-}811}$	+	-
$HCF\text{-}1_{750\text{-}806}$	++	-
$HCF\text{-}1_{750\text{-}801}$	+/-	-
$HCF\text{-}1_{750\text{-}796}$	-	-
$HCF\text{-}1_{770\text{-}836}$	+++	-
$HCF\text{-}1_{790\text{-}836}$	-	-
$HCF\text{-}1_{790\text{-}902}$	++	++

B

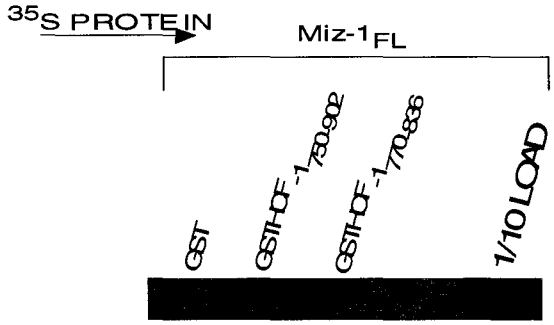


Figure 3.15: Fine mapping to determine HCF-1 residues involved in binding to Miz-1 and GABP. A. Yeast 2-hybrid assay was utilized to detect interaction between HCF-1 constructs and Miz-1 or GABP. Yeast strain, Y190, was transformed with the indicated plasmids and grown on appropriate media for 3-4 days. β-gal activity was determined qualitatively using β-gal overlay assay with X-gal as substrate. Each plus sign represents 25% of full activity (or 25% of the strongest interaction) as estimated from the intensity of blue. B. *In vitro* GST pull-down assay. ^{35}S-methionine labelled Miz-1 synthesized *in vitro* was incubated with GST alone or with various GST-fusion proteins as indicated, and bound material was analysed by SDS- polyacrylamide gel electrophoresis. The 1/10 load lanes represent 10% of the ^{35}S-methionine labelled protein added to the respective binding assays.

generated a GST fusion to HCF-1 residues 770-836 and tested binding to radiolabelled Miz-1 in GST pull-down assays. As shown in figure 3.15b, radiolabelled full length Miz-1 bound to a GST fusion to HCF-1 residues 750-902 but not to GST alone, as shown previously in figure 3.4. In addition, radiolabelled full length Miz-1 retained interaction with a GST fusion to HCF-1 residues 770-836. These results confirm that HCF-1 residues 770-836 are sufficient to bind to Miz-1 and taken together with results presented in figure 3.15a, suggest that these residues are selective in their interaction with Miz-1. Interestingly, since these experiments were conducted, a third protein, Sin3 HDAC, has been shown to interact with the HCF-1 basic domain (Wysocka *et al.*, 2003). However, this interaction has been mapped to HCF-1 residues 610-722 (Wysocka *et al.*, 2003) and therefore we believe residues 770-836 are selective for Miz-1, although there may exist additional HCF-1 basic domain partners that are not known at this time that may also target these residues.

Thus, to date, we have discovered an HCF-1 fragment that selectively interacts with Miz-1. Our objective was to determine whether this HCF-1 fragment would have any effect on the rescue of the tsBN67$_{HR1}$ temperature-sensitive cellular proliferation defect by HCF-1$_{1-1011}$, thus indicating the importance of Miz-1 to HCF-1-mediated cellular proliferation. To accomplish this, tsBN67$_{HR1}$ cells were transfected with pSV2-NEO, or HCF-1$_{1-1011}$, in combination with HCF-1$_{750-902}$ (a fragment that binds to both Miz-1 and GABP), HCF-1$_{770-836}$ (a fragment that selectively binds Miz-1), HCF-1$_{1-380}$ (the kelch domain that interacts with a number of cellular proteins), or the HCF-1 empty vector as shown in figure 3.16. Curiously, rescue by HCF-1$_{1-1011}$ was unaffected by co-

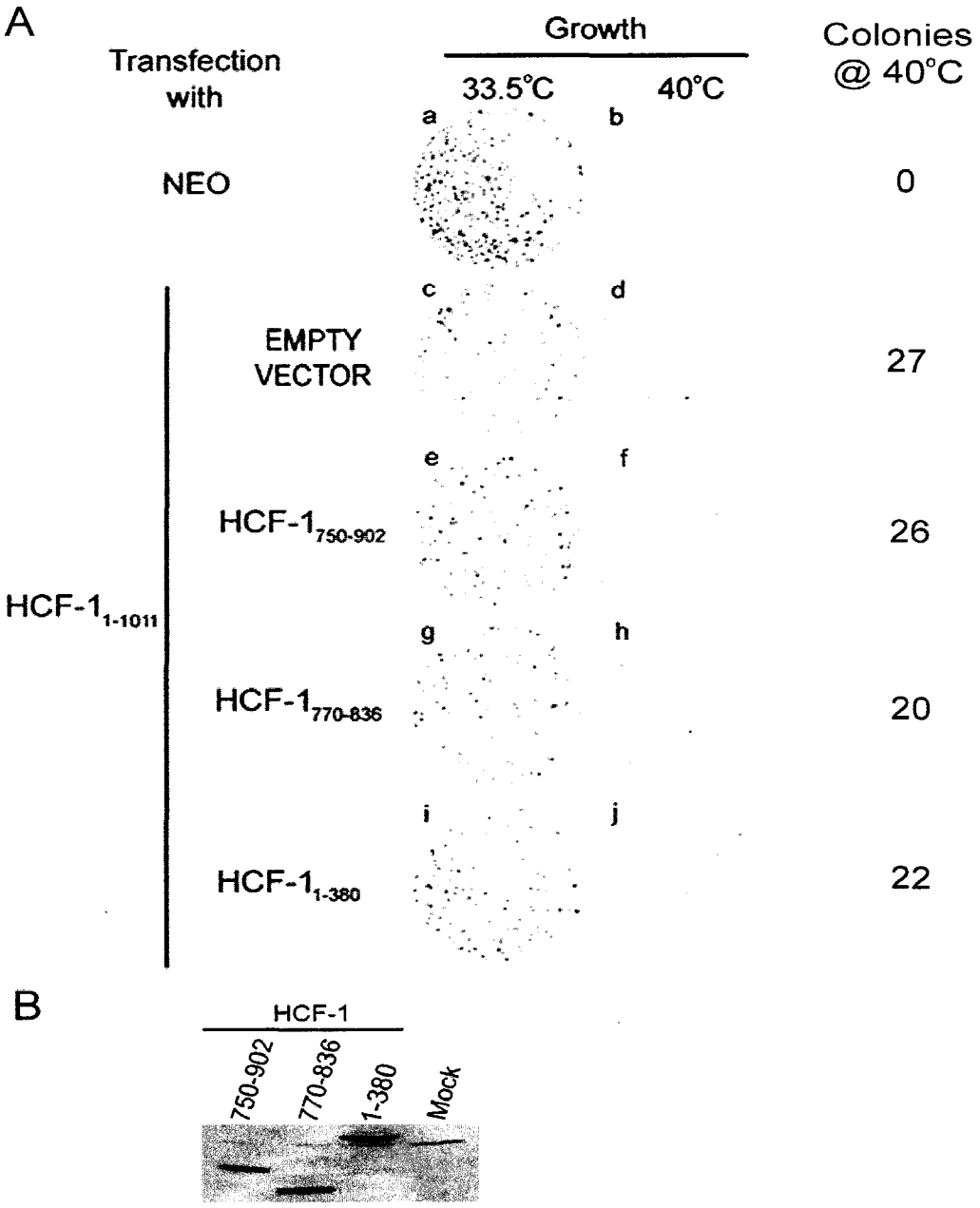


Figure 3.16: HCF-1 fragments do not prevent HCF-1-mediated rescue of the temperature-sensitive cellular proliferation defect in tsBN67$_{HR1}$ cells. A. tsBN67$_{HR1}$ cells were transfected with pSV2-NEO, or HCF-1$_{1-1011}$, in conjunction with Flag-tagged fusions of HCF-1 or empty vector, as shown. Cells were grown at 33.5°C or 40°C under G418 selection for 3 weeks and visualized by staining with crystal violet. Colonies @ 40°C represents the number of colonies visible on the plate following 3 weeks of growth from the representative experiment. B. The expression levels of Flag-tagged HCF-1 fusions were determined by Western blotting with anti-Flag antibody. Lysates were produced from tsBN67$_{HR1}$ cells transfected with 2.0μg of the expression vector for the indicated HCF-1 construct. The results show representative plates from an experiment performed three times.

transfection of any of the HCF-1 fragments (figure 3.16a; compare plates b, d, f, h, and j). However, it is difficult to predict the outcome of overexpressing fragments of HCF-1 that block the interaction of endogeneous full length HCF-1 and its endogeneous interaction partners in $tsBN67_{HR1}$ cells. For instance the kelch domain interacts with a large number of cellular factors and since HCF-1 may be both a co-activator and a co-repressor, overexpression of the kelch domain fragment may deregulate the expression of both growth-inhibitory and growth-promoting genes, with the effects cancelling each other out. In addition, the HCF-1 fragments may interact with additional proteins which we are unaware of and influence unknown target genes, making it difficult to predict the outcome of their overexpression. Lastly, the effect of the overexpressed HCF-1 fragments may be concentration-dependent in $tsBN67_{HR1}$ cells, and thus the amounts expressed in these experiments may be suboptimal. Western blots are shown in figure 3.16b demonstrating that although these HCF-1 fragments did not affect rescue, they were expressed in $tsBN67_{HR1}$ cells following transfection. These results indicate that the subfragments of HCF-1 used here are unable to affect rescue by $HCF\text{-}1_{1\text{-}1011}$ unlike the C-terminal fragment of HCF-1 used by Sharp and colleagues (Scarr and Sharp, 2002).

3.2.5 The HCF-2 Fn3 repeats interact with HCF-1 partners including Miz-1

Human cells contain an HCF-1-like protein, termed HCF-2 (Johnson *et al.*, 1999), which shares with HCF-1 conserved amino- and carboxy-terminal domains (Johnson *et al.*, 1999). Although HCF-2 is able to support VP16-induced complex (VIC) formation, it is unable to rescue the temperature-sensitive cell cycle defect in $tsBN67_{HR1}$ cells (Johnson *et al.*, 1999; Lee and Herr, 2001). Interestingly, rescue by HCF-1 is inhibited

by co-expression of HCF-2 (Johnson *et al.*, 1999). This suggests that HCF-1 and HCF-2 represent a pair of regulators of cell proliferation that counteract each other. It has been suggested that HCF-2 antagonizes HCF-1 activity through binding to HCF-1 interaction partners, and displaying different activities with shared partners, such as with VP16 (Johnson *et al.*, 1999; Lee and Herr, 2001). HCF-2 shares with HCF-1 both the amino-terminal kelch domain and the carboxy-terminal Fn3 repeats, both of which fold into protein-protein interaction surfaces (Johnson *et al.*, 1999; Wilson *et al.*, 2000). The presence of Fn3 repeats in HCF-2 has been particularly puzzling since these repeats in HCF-1 serve as self-association sequences allowing for non-covalent re-association of amino- and carboxy-terminal fragments following proteolytic cleavage of HCF-1 (Wilson *et al.*, 2000). Since HCF-2 is not proteolytically processed, the presence of Fn3 repeats in HCF-2 may indicate a dual role for these sequences in HCF-1 function that is conserved in HCF-2 (Wilson *et al.*, 2000). Thus, it has been hypothesized that in HCF-2, the Fn3 repeats may serve as a protein-protein interaction surface with cellular proteins (Wilson *et al.*, 2000).

Although a small fragment of HCF-1 which selectively bound Miz-1 did not influence HCF-$1_{1\text{-}1011}$ complementation of tsBN67 cells, we sought to determine whether HCF-2 interacted with any HCF-1 interaction partners and whether any of these interactions contributed to its antagonistic effect on HCF-1 complementation of tsBN67 cells. These experiments were designed to define regions of HCF-2 and interactions that are important for its function, and indirectly assess the importance of shared interaction partners in HCF-1-mediated cellular proliferation. Full length HCF-2 fused to GAL4-

DBD has been shown by Lee and Herr, and in our own observations, to be non-functional in yeast 2-hybrid assays even though it is expressed (Lee and Herr, 2001). However, various HCF-2 fusions to GAL4-DBD were constructed and these were tested for interaction by yeast 2-hybrid analysis with GAL4-AD-Miz-1, -GABP, -Zhangfei (ZF), and –VP16. As shown in figure 3.17, the amino-terminal residues 2-373, which forms the kelch domain in HCF-2 (Johnson *et al.*, 1999), only bound Zhangfei, and not with VP16. The central region of HCF-2 corresponding to residues 373-596, which lacks any homology to HCF-1 (Johnson *et al.*, 1999) failed to interact with any of the proteins tested here. By contrast, the carboxy-terminal domain of HCF-2 which is homologous to the Fn3 repeats in HCF-1 (Johnson *et al.*, 1999; Wilson *et al.*, 2000) interacted with both Miz-1 and GABP. In an attempt to further define subfragments within the carboxy-terminal HCF-2 Fn3 repeats that would selectively bind either Miz-1 or GABP, additional mutants were constructed as GAL4-DBD fusions. As shown in figure 3.17, residues 662-792 binds to GABP selectively, whereas residues 727-792 binds to Miz-1 selectively. These results have identified Miz-1, GABP, and Zhangfei as common interaction partners that are shared between HCF-1 and HCF-2, and furthermore, that the Fn3 repeats in HCF-2 do function as a protein-protein interaction surface, recruiting Miz-1 and GABP. In order to confirm the Miz-1/HCF-2 interaction we utilized the *in vitro* GST pull-down assay with a variety of HCF-2 GST fusions and radiolabelled Miz-1, or a GST fusion to Miz-1 residues 637-803 and radiolabelled HCF-2 fragments. As shown in figure 3.18, radiolabelled full length HCF-2 interacted with GST-Miz-$1_{637\text{-}803}$ but not to GST alone confirming an interaction between HCF-2 and Miz-1. In agreement with the

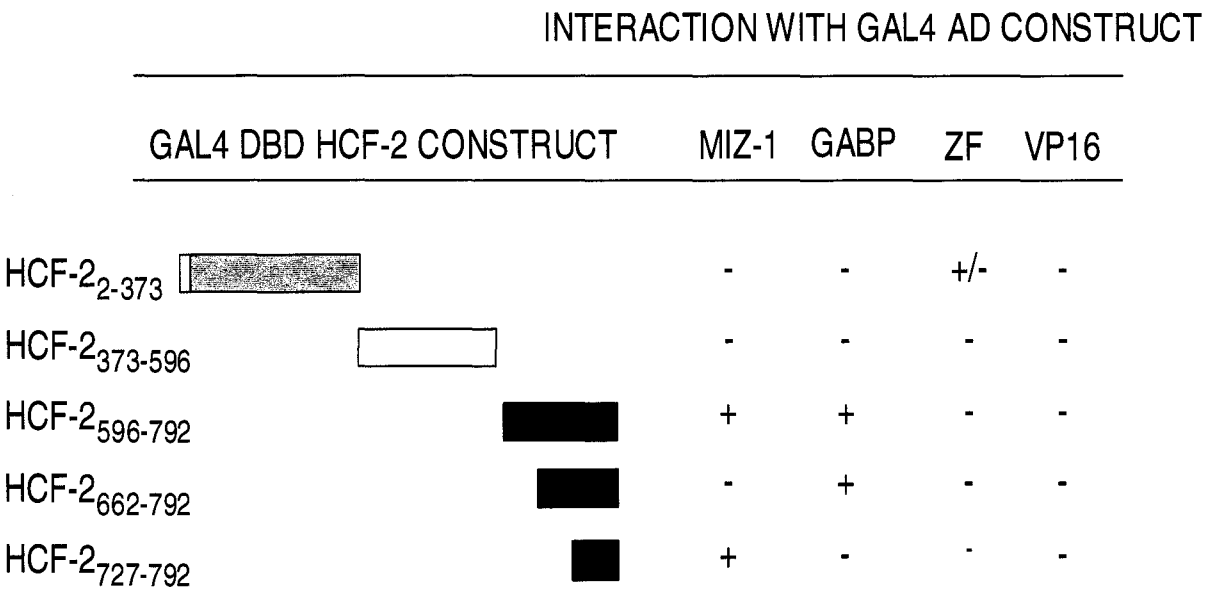


Figure 3.17: The HCF-2 Fn3 repeats interact with HCF-1 partners. Yeast 2-hybrid assay was utilized to detect interaction between HCF-2 constructs and various HCF-1 interaction partners as shown. Yeast strain Y190 was transformed with yeast expression vectors for the indicated proteins and grown on appropriate media for 3-4 days. β-gal activity was determined qualitatively using the β-gal overlay assay with X-gal as substrate. Interaction is indicated by (+).

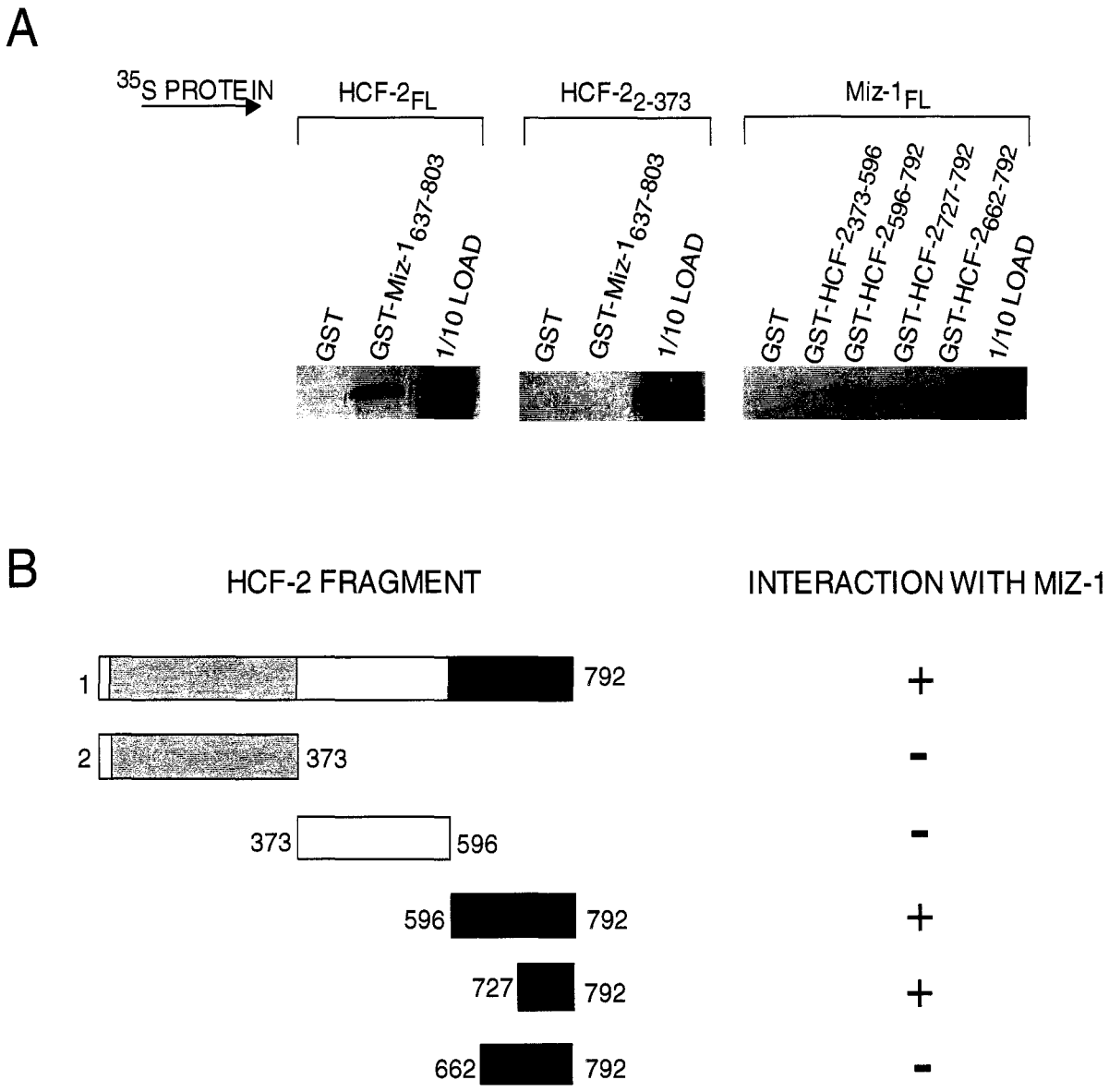


Figure 3.18: HCF-2 residues 727-792 are sufficient for Miz-1 interaction. A. *In vitro* GST pull-down assay. A variety of ^{35}S-methionine labelled polypeptides synthesized *in vitro* were incubated with GST alone or with various GST-fusion proteins as indicated, and bound material was analysed by SDS- polyacrylamide gel electrophoresis. The 1/10 load lanes represent 10% of the ^{35}S-methionine labelled protein added to the respective binding assays. B. Schematic representation of the HCF-2 deletion constructs used above and summary of the *in vitro* HCF-2 interaction results. +, specific interaction; -, no interaction.

results from the yeast 2-hybrid analysis, a radiolabelled fragment of HCF-2 corresponding to its kelch domain failed to interact with GST-Miz-$1_{637-803}$. Furthermore, radiolabelled full length Miz-1 interacted with both GST-HCF-$2_{596-792}$ and GST-HCF-$2_{727-792}$ but failed to interact with GST-HCF-$2_{373-596}$ and GST-HCF-$2_{662-792}$ in agreement with yeast 2-hybrid results presented in figure 3.17. These results are summarized in figure 3.18b.

3.2.6 A fragment of HCF-2 encompassing residues 662-792, which selectively binds GABP, is sufficient to reduce rescue of the tsBN67 cell cycle defect by HCF-1_{1-1011}

Although HCF-2 cannot functionally replace HCF-1 in rescuing the tsBN67 cellular proliferation defect, some overlap in function is evident by the fact that co-expression of HCF-2 prevents HCF-1-mediated rescue of the temperature-sensitive defect in tsBN67 cells (Johnson *et al.*, 1999). We sought to determine which domains of HCF-2 retain this trans-dominant inhibitor effect on cell cycle rescue by HCF-1. To determine which, if any, domains of HCF-2 retain the ability of full length HCF-2 to prevent HCF-1-mediated cellular proliferation in tsBN67_{HR1} cells, the effect of co-transfection of various HCF-2 fragments on complementation by HCF-1 was determined. Unfortunately, the effect of an HCF-2 fragment, which selectively binds to Miz-1 (HCF-2 residues 727-792), on complementation by HCF-1 remains undetermined since such a fragment could not be stably expressed in tsBN67_{HR1} cells following transfection. However, we were able to test the effect of three HCF-2 fragments, a kelch domain fragment (HCF-2 residues 2-373), the central region which lacks homology to HCF-1

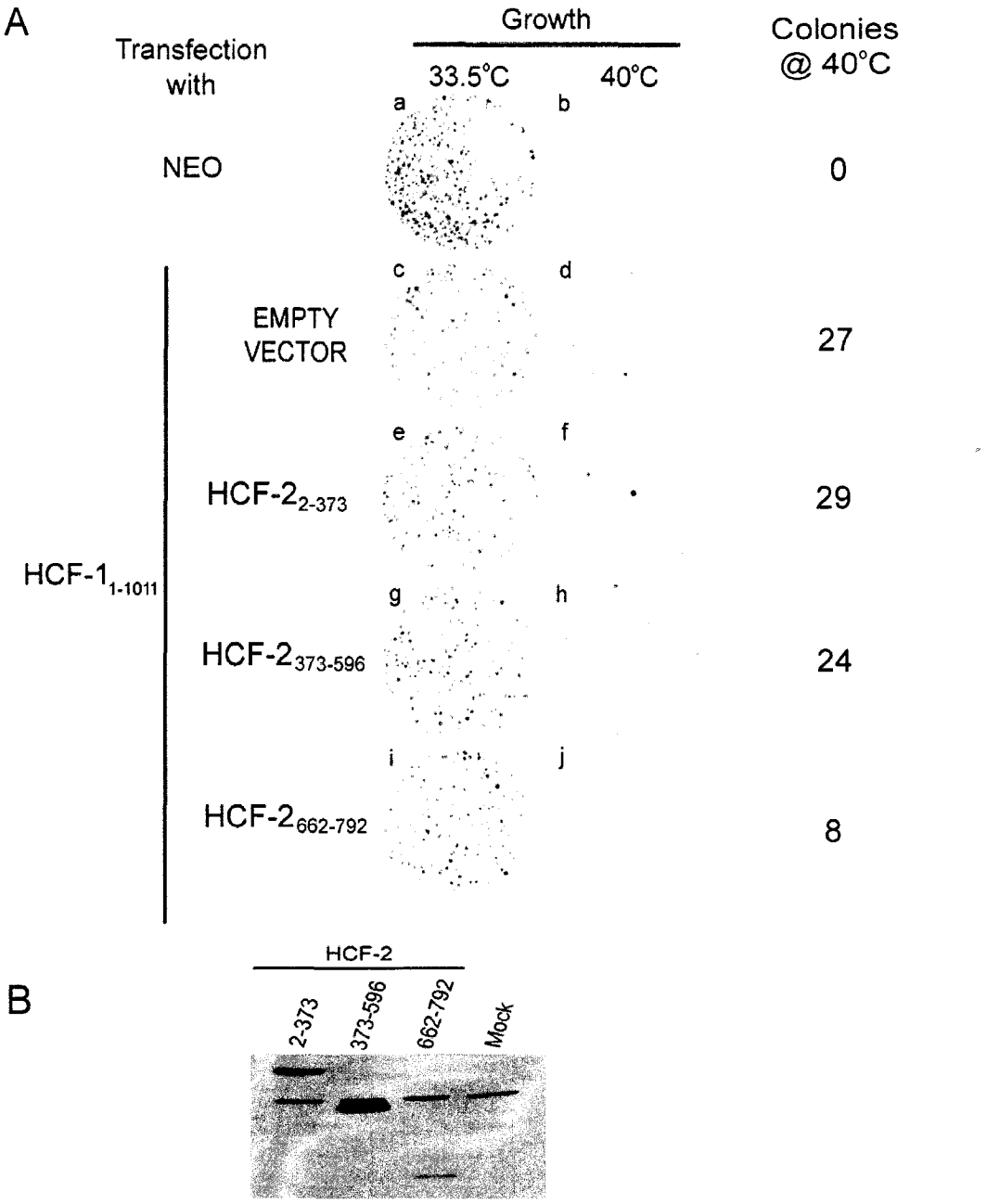


Figure 3.19: A fragment of HCF-2 encompassing residues 662-792, which selectively binds GABP, is sufficient to reduce HCF-1-mediated rescue of tsBN67 cells. A. $tsBN67_{HR1}$ cells were transfected with pSV2-NEO, or $HCF\text{-}1_{1\text{-}1011}$, in conjunction with Flag-tagged fusions of HCF-2 or empty vector, as shown. Cells were grown at 33.5°C or 40°C under G418 selection for 3 weeks and visualized by staining with crystal violet. Colonies @ 40°C represents the number of colonies visible on the plate following 3 weeks of growth from the representative experiment. B. The expression levels of Flag-tagged HCF-2 fusions were determined by Western blotting with anti-Flag antibody. Lysates were produced from $tsBN67_{HR1}$ cells transfected with 2.0μg of the expression vector for the indicated HCF-2 constructs. The results show representative plates from an experiment performed three times.

(HCF-2 residues 373-596), and the carboxy-terminal region (HCF-2 residues 662-792) which selectively binds GABP. Thus, tsBN67$_{HR1}$ cells were transfected with pSV2-NEO, or HCF-1$_{1-1011}$, in conjunction with HCF-2$_{2-373}$, HCF-2$_{373-596}$, HCF-2$_{662-792}$, or the HCF-2 empty vector as shown in figure 3.19. Co-transfection of either HCF-2$_{2-373}$ or HCF-2$_{373-596}$ in conjunction with HCF-1$_{1-1011}$ did not have an appreciable effect on rescue by HCF-1$_{1-1011}$ (compare plates d, f, and h) suggesting that these fragments do not interact with any cellular factors required by HCF-1 for rescue. By contrast, co-transfection of HCF-2$_{662-792}$, which selectively binds GABP, in conjunction with HCF-1$_{1-1011}$ greatly reduced colony formation (compare plates d and j) suggesting that the interaction between HCF-2 and GABP may sequester GABP from HCF-1 thereby preventing rescue by HCF-1. A Western blot is shown in figure 3.19b demonstrating expression levels of the HCF-2 fragments in tsBN67$_{HR1}$ cells following transfection. These results are consistent with the results from the HCF-1 mutagenesis complementation experiments, in which a mutant of HCF-1 reduced in its ability to bind GABP was less efficient in complementing tsBN67 cells than wild type HCF-1, and suggest that the HCF-1 interaction with GABP is required for HCF-1-mediated cell cycle progression.

3.3 IDENTIFYING AND CHARACTERIZING HBM-CONTAINING KELCH DOMAIN INTERACTION PARTNERS

3.3.1 The E2F-4 primary sequence contains an HCF-binding motif (HBM)

A plethora of HCF-1-interacting proteins have been described; however, the identity of cellular factors contributing to HCF-1-mediated cell cycle progression remain largely undiscovered. Since the kelch domain is required for cell cycle progression, in addition to the basic domain (Wilson *et al.*, 1997), we sought to discover kelch domain

interacting proteins to provide insights into the mechanisms of action of HCF-1. To this end a yeast 2-hybrid screen with the HCF-1 kelch domain as bait was conducted. Those experiments yielded only previously identified HCF-1 interaction partners; Luman and Zhangfei (Freiman and Herr, 1997; Lu *et al.*, 1997; Lu and Misra, 2000a). Consequently, another approach was utilized whereby we conducted a database search for cellular proteins containing an HCF-binding motif, a motif that has been shown to mediate interaction with the HCF-1 kelch domain (Freiman and Herr, 1997; Lai and Herr, 1997; Lu *et al.*, 1998; Lu and Misra, 2000a; Mahajan *et al.*, 2002a).

The modular nature of the HCF-1 protein is shown in figure 1.5a. The amino-terminal kelch domain interacts with a variety of cellular factors as well as the viral protein VP16 (Wysocka and Herr, 2003). These proteins contain an HBM (figure 3.20a) which targets the kelch domain. Thus, a database search was conducted which has revealed that a variety of additional cellular factors, known to play a role in transcriptional control and cellular proliferation, contain an HBM (figure 3.20b). Most intriguing, was the discovery that 2 members of the E2F family of transcription factors, E2F-1 and E2F-4, known to play a central role in cellular proliferation (DeGregori, 2002; Trimarchi and Lees, 2002), contain an HBM. Schematic diagrams of E2F-1 and E2F-4, indicating the location of the HBM are presented in figure 3.21. The E2F-1 HBM, DHQY, is located at amino acids 96-99 embedded within a domain that interacts with cyclinA/CDK2 (Krek *et al.*, 1994; Xu *et al.*, 1994) and adjacent to the nuclear localization sequence (Muller *et al.*, 1997; Verona *et al.*, 1997). The E2F-4 HBM, DHDY, is located at amino acids 389-392 embedded within the transactivation domain

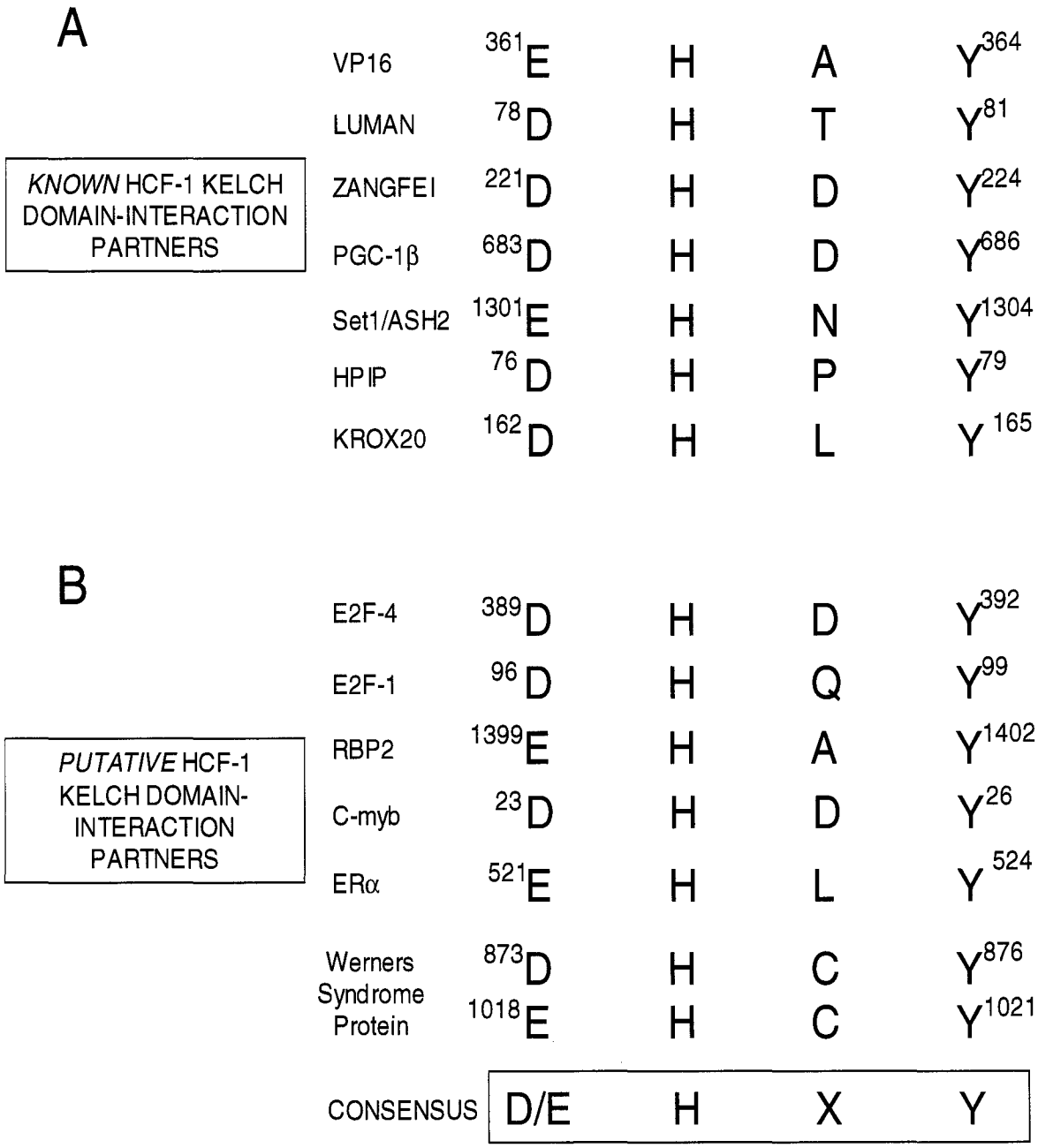


Figure 3.20: E2F-1 and E2F-4 contain an HBM (HCF-Binding Motif). A. List of various proteins known to interact with the HCF-1 kelch domain through an HBM. Letters indicate single amino acid codes and numbers refer to the amino acid coordinates within each protein. B. List of various cell cycle proteins discovered in the database search described here for proteins containing a putative HBM. Letters indicate single amino acid codes and numbers refer to the amino acid coordinates within each protein. The database search was conducted by J. Knez.

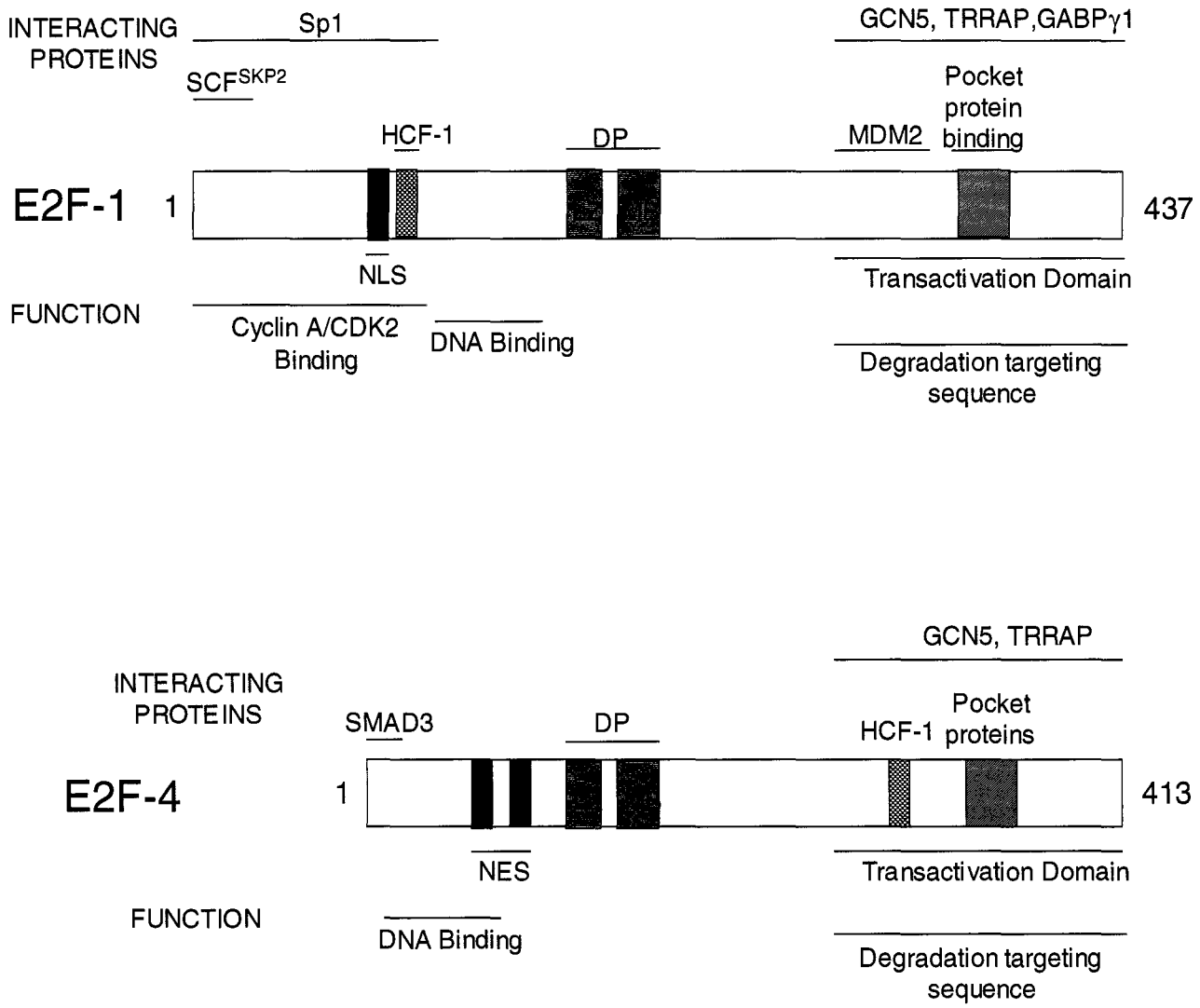


Figure 3.21: Schematic diagrams of E2F-1 and E2F-4. Diagrams depict the modular domain structure of E2F-1 and E2F-4 and associated functions and interacting proteins. E2F-1, a member of the 'activating E2Fs', is similar in structure to E2F-4, a member of the 'repressive E2Fs' except for some differences: E2F-4 contains NES sequences whereas E2F-1 contains an NLS; E2F-1 has a domain N-terminal to the DNA-binding domain known as the cyclin A/CDK2-binding domain. The HBM in E2F-4 is embedded within the transactivation domain and is adjacent to the pocket protein binding domain. The HBM in E2F-1 is located on the periphery of the cyclin A/cdk2-binding domain and is adjacent to the NLS.

and adjacent to the pocket protein binding site (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Sardet *et al.*, 1995; Lang *et al.*, 2001).

The E2F family of transcription factors, which belong to the basic helix-loop-helix class of transcription factors, can be divided into 3 distinct subgroups on the basis of their sequence and structural similarities, as well as their functional roles (Trimarchi and Lees, 2002). The first group consists of E2F-1-3 and are termed the 'activating E2Fs'. The key role for these E2Fs is the activation of genes that are essential for cellular proliferation (DeGregori *et al.*, 1995a; Ishida *et al.*, 2001; Muller *et al.*, 2001; Wu *et al.*, 2001). Overexpression of any of these proteins is sufficient to induce quiescent cells to re-enter the cell cycle (Johnson *et al.*, 1993; Shan *et al.*, 1996; DeGregori *et al.*, 1997), lead to transformation of primary cells (Singh *et al.*, 1994; Conner *et al.*, 2000), and overcome growth arrest signals (DeGregori *et al.*, 1995a; DeGregori *et al.*, 1995b; Schwarz *et al.*, 1995). E2F-1 also appears to have a unique function in that it activates genes that contribute to apoptosis (DeGregori *et al.*, 1997; Nahle *et al.*, 2002). In support of this, E2F-1 has been shown to cause both increased proliferation and apoptosis in *in vitro* studies (DeGregori *et al.*, 1997; Nahle *et al.*, 2002), and E2F-1 deficient mice develop tumours (Yamasaki *et al.*, 1996), which is consistent with a role for E2F-1 in apoptosis as well as proliferation.

By contrast, E2F-4/5 seem to be primarily involved in the active repression of E2F responsive genes at G0/G1 through recruitment of pocket proteins and associated chromatin remodeling activities, and are thus termed the 'repressive E2Fs' (Tommasi and Pfeifer, 1995; Vairo *et al.*, 1995; Li *et al.*, 1997; Iavarone and Massague, 1999;

Takahashi *et al.*, 2000; Trimarchi and Lees, 2002). Indeed, the majority of the evidence suggests that E2F-4 is responsible for cell cycle arrest or exit, and differentiation through its ability in G0/G1 to recruit pocket proteins and associated HDAC complexes to certain gene promoters (Paramio *et al.*, 2000; Takahashi *et al.*, 2000). Specifically, E2F-4/5 deficient mouse embryo fibroblasts (MEFs) failed to arrest in G1 in response to $p16^{INK4a}$ (Gaubatz *et al.*, 2000). Alternatively, under certain experimental conditions E2F-4 can also lead to cellular proliferation and display oncogenic properties (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994; Wang *et al.*, 2000). In support of this, E2F-4, like E2F-1, has been shown to contain an autonomous transactivation domain in its C-terminus that binds the acetyltransferase GCN5 and cofactor TRAPP *in vivo* (Lang *et al.*, 2001). Thus, although it is hypothesized that E2F-1-3 are transcriptional activators and cell cyle promotors, whereas E2F-4/5 are transcriptional repressors and cell cycle inhibitors, E2F-4 contains a transactivation domain and thus may stimulate transcription in a restricted developmental or cell cycle window at certain target promoters (Lang *et al.*, 2001; Pierce *et al.*, 1998).

3.3.2 E2F-1 and E2F-4, but not E2F-5, interact with HCF-1 in mammalian cells

The E2F family of transcription factors contains 7 members; E2F-1 through E2F-7. Thus, of the 7 E2F family members, only E2F-1 and E2F-4 contain a putative HBM. To determine whether these E2F factors could indeed bind to HCF-1 in mammalian cells *in vivo*, J. Knez carried out co-immunoprecipitation experiments with $tsBN67_{HR1}$ cells co-transfected with expression vectors for HA-tagged E2Fs and Flag-tagged HCF-1_{1-902}. Cell extracts were immunoprecipitated with anti-Flag antibody. The precipitates were

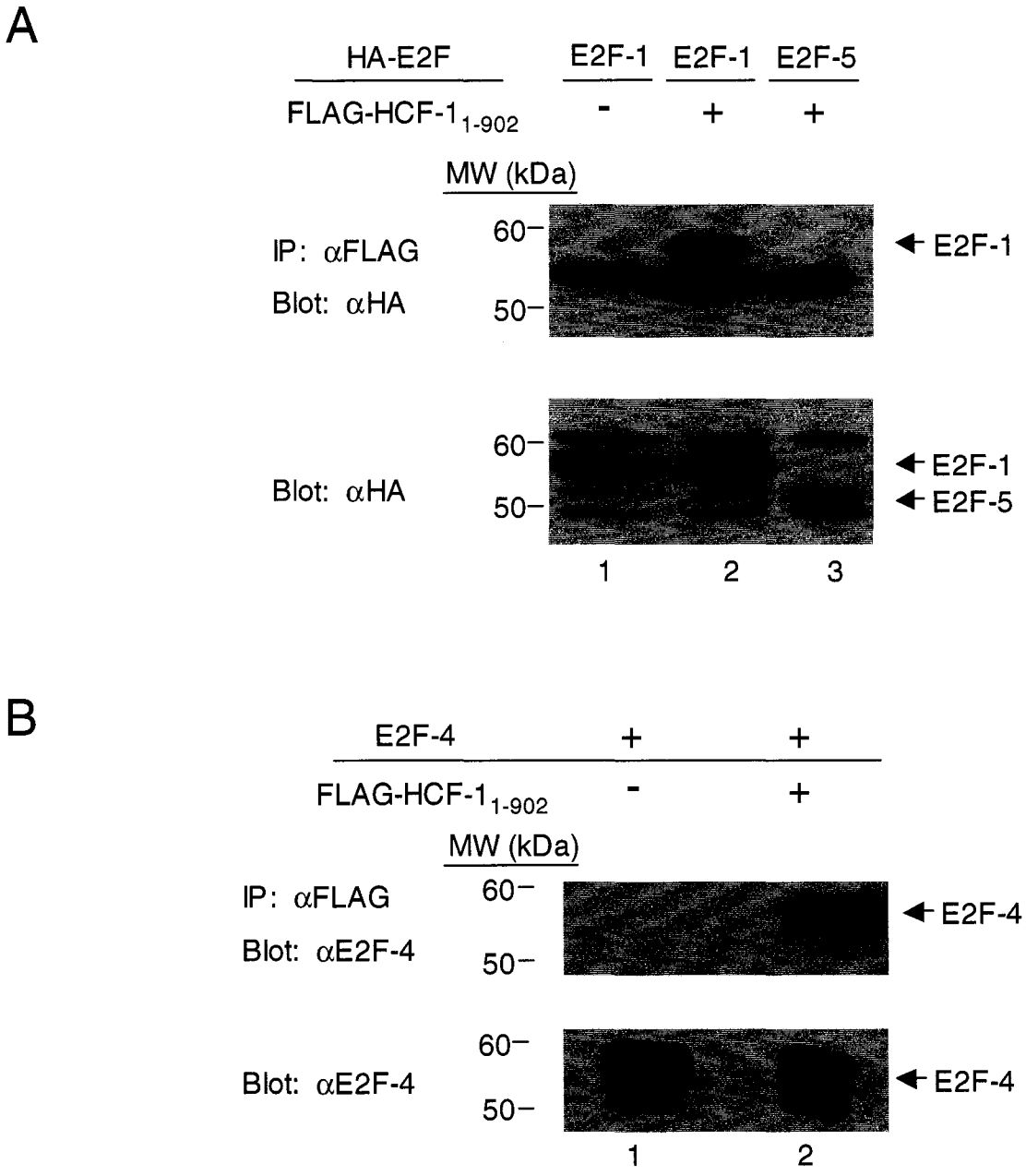


Figure 3.22: E2F-1 and E2F-4, but not E2F-5, interact with HCF-1. A. E2F-1, but not E2F-5 interacts with HCF-1. tsBN67$_{HR1}$ cells were transfected with expression vectors for Flag-HCF-1$_{1-902}$ and HA-E2F-1/5 or HA-E2F-1 alone as indicated. Cell extracts were immunoprecipitated (IP) with anti-Flag antibody and the precipitates were resolved on a SDS-polyacrylamide gel and probed with anti-HA antibody to detect HA-E2Fs (top panel). To monitor protein expression, extracts were blotted directly using anti-HA antibody (bottom panel). This panel represents the work of J. Knez, except the Western blotting. B. E2F-4 interacts with HCF-1. tsBN67$_{HR1}$ cells transfected with expression vectors for HA-E2F-4 and Flag-HCF-1$_{1-902}$ or HA-E2F-4 alone, as indicated, Cell extracts were immunoprecipitated (IP) with anti-Flag antibody and the precipitates were resolved on a SDS-polyacrylamide gel and probed with anti-E2F-4 antibody to detect HA-E2F-4 (top panel). To monitor protein expression, extracts were blotted directly using anti-E2F-4 antibody (bottom panel). This panel represents the work of J. Knez.

resolved on a SDS polyacrylamide gel and probed with anti-HA, or anti-E2F-4 antibody. As shown in figure 3.22a, HA-E2F-1 was present in immune complexes precipitated with anti-Flag antibody from cells that had been transfected with both Flag-HCF-$1_{1\text{-}902}$ and HA-E2F-1 expression vectors, but not from control cells that were transfected with HA-E2F-1 alone (compare lanes 1 and 2). In contrast to E2F-1, HA-E2F-5, which lacks the HBM, failed to co-immunoprecipitate with Flag-HCF-$1_{1\text{-}902}$. Similar to the result with E2F-1, figure 3.22b demonstrates that E2F-4 was present in immune complexes precipitated with anti-Flag antibody from cells that had been transfected with both Flag-HCF-$1_{1\text{-}902}$ and HA-E2F-4 expression vectors, but not from control cells that were transfected with HA-E2F-4 alone (compare lanes 1 and 2). These results show that E2F factors that contain an HBM, namely E2F-1 and E2F-4, can form a complex in mammalian cells with HCF-1.

3.3.3 HCF-1_{FL} interacts with both E2F-4_{WT} and an HBM mutant version of E2F-4

We chose to focus our attention on E2F-4 since it is expressed at higher levels than the other E2Fs and accounts for at least half of the *in vivo* E2F activity (Vairo *et al.*, 1995; Moberg *et al.*, 1996). Furthermore, E2F-4 is a 'repressive E2F' which is thought to play an integral role in cell cycle control at the G0/G1 stage (Paramio *et al.*, 2000; Takahashi *et al.*, 2000), the stage at which tsBN67 cells arrest (Goto *et al.*, 1997). To examine the role of the putative HBM in E2F-4 binding to HCF-1, we generated a mutation within the HBM whereby the critical tyrosine was changed to alanine. A similar mutation has been shown to disrupt HBM-dependent binding between HCF-1 and other HBM-containing proteins (Freiman and Herr, 1997; Lu *et al.*, 1998; Lu and Misra,

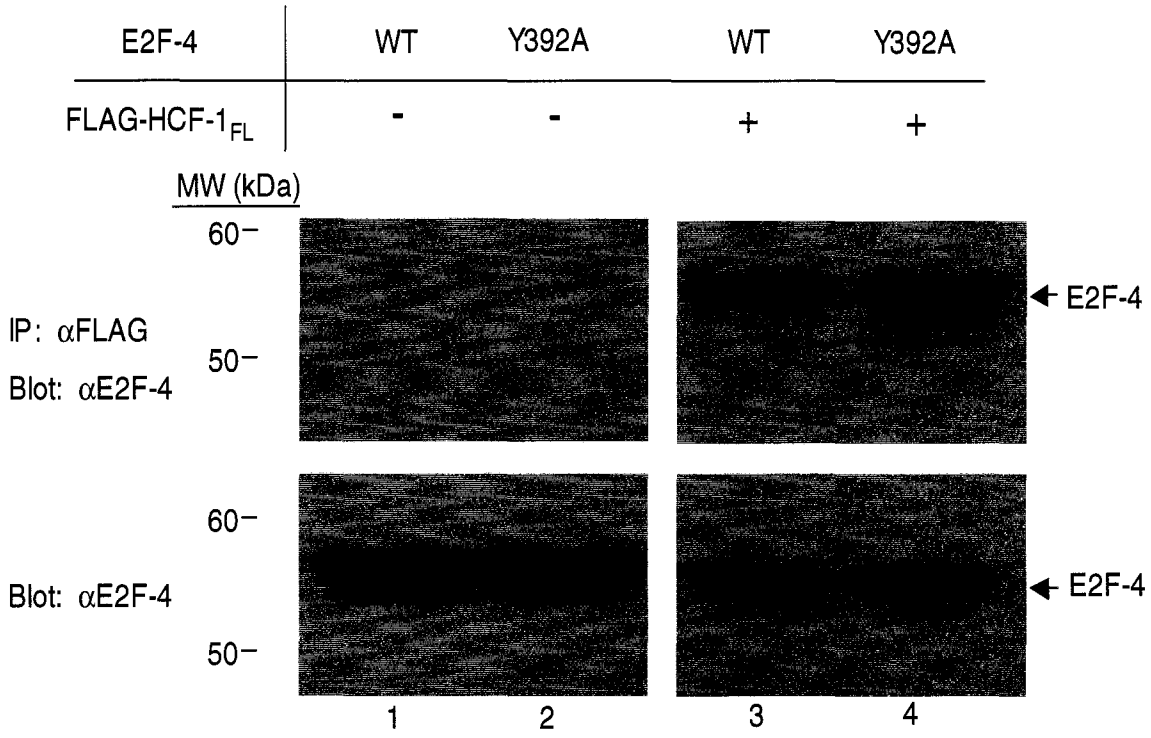


Figure 3.23: HCF-1_{FL} interacts with wild type E2F-4, as well as an HBM mutant version. tsBN67$_{HR1}$ cells were transfected with expression vectors for Flag-HCF-1_{FL} and wild type E2F-4 or an HBM mutant version, E2F-4_{Y392A}, or E2F-4 vectors alone, as indicated. Cell extracts were immunoprecipitated (IP) with anti-Flag antibody and the precipitates were resolved on a SDS-polyacrylamide gel and probed with anti-E2F-4 antibody to detect HA-E2F-4 (top panel). To monitor protein expression, extracts were blotted directly using anti-E2F-4 antibody (bottom panel). These experiments were done in collaboration with J. Knez.

2000a). As shown in figure 3.23, co-immunoprecipitation of E2F-4_{WT} was dependent on co-expression of Flag-HCF-1_{FL} (compare lanes 1 and 3). Surprisingly, the HBM mutant, E2F-4_{Y392A}, retained binding to full length HCF-1 and was dependent on co-expression of Flag-HCF-1_{FL}. We postulated that this result could indicate that another region in E2F-4 contacts a domain within full length HCF-1, and this set of contacts is sufficient to mediate E2F-4/HCF-1 interaction, in addition to the HBM/kelch domain interaction contact. Additional experiments were conducted to test this hypothesis.

3.3.4 E2F-4 interacts with both the HCF-1 kelch and basic domains – HBM mutant retains binding to basic domain but loses interaction with kelch domain

If HCF-1 and E2F-4 each contained 2 sets of interaction motifs then we expected that the HBM mutant version of E2F-4 would fail to bind to the HCF-1 kelch domain but retain binding to the basic domain. To test this possibility, co-immunoprecipitation experiments were conducted between the kelch (HCF-1_{1-380}) and basic (HCF-$1_{450-902}$) domains and both wild type E2F-4_{WT} and HBM mutant E2F-4 (E2F-4_{Y392A}). E2F-4_{WT} co-immunoprecipitated with both the HCF-1 kelch and basic domains (compare lanes 1 and 3, figure 3.24) supporting the hypothesis that HCF-1 contains 2 domains sufficient to interact with E2F-4. Furthermore, in contrast to wild type E2F-4, the HBM mutant failed to co-immunoprecipitate with the HCF-1 kelch domain (compare lanes 1 and 2) indicating that E2F-4 contains a *bona fide* HBM. In addition, the E2F-4 HBM mutant retained binding to the HCF-1 basic domain (compare lanes 3 and 4). Taken together, these results show that HCF-1 and E2F-4 can form a complex *in vivo* and that this interaction is mediated by 2 sets of contacts: E2F-4 interacts with the HCF-1 kelch

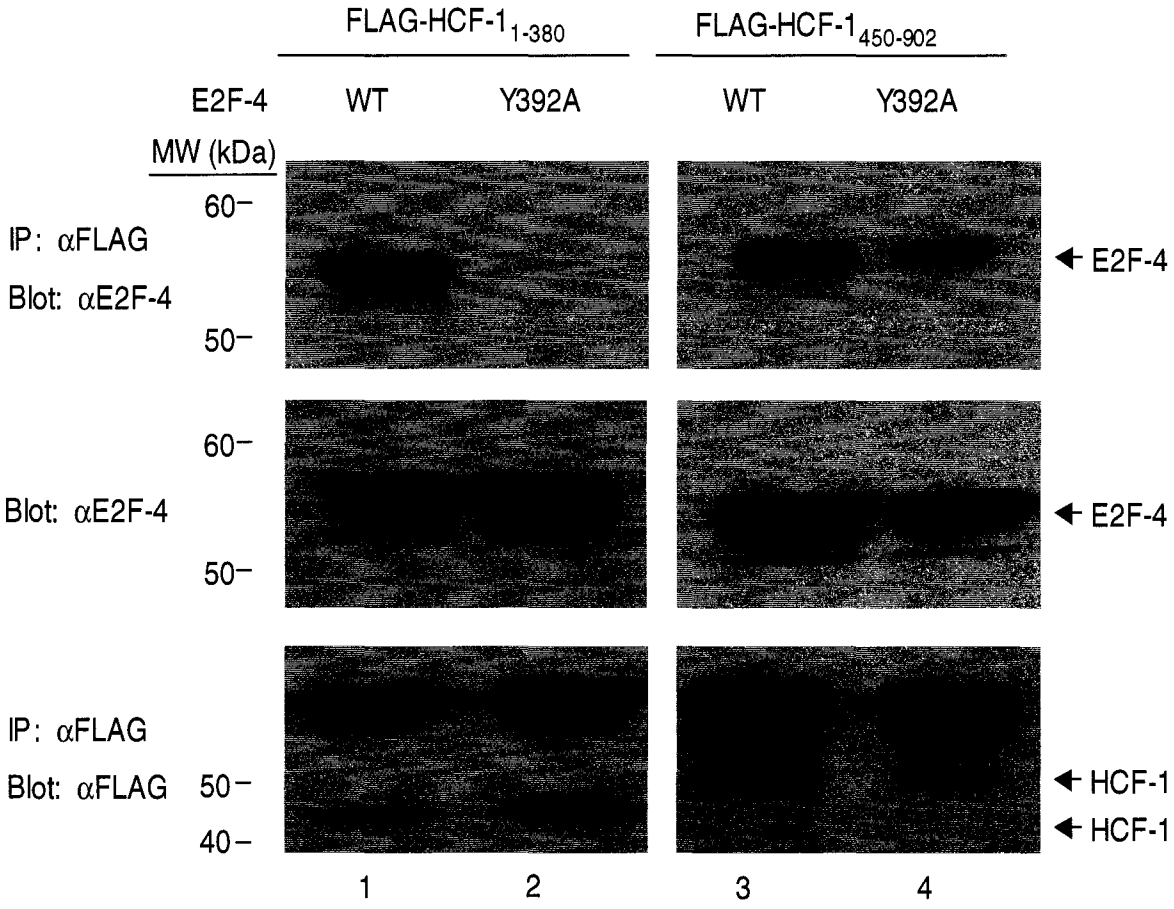


Figure 3.24: E2F-4 interacts with both the HCF-1 kelch and basic domains – HBM mutant retains binding to basic domain but loses interaction with kelch domain. tsBN67$_{HR1}$ cells were transfected with expression vectors for Flag-HCF-1$_{1-380}$ or Flag-HCF-1$_{450-902}$, along with either wild type E2F-4 or E2F-4$_{Y392A}$ as indicated. Cell extracts were immunoprecipitated (IP) with anti-Flag antibody and the precipitates were resolved on a SDS-polyacrylamide gel and probed with anti-E2F-4 antibody to detect HA-E2F-4 (top panel). To monitor protein expression, extracts were blotted directly using anti-E2F-4 antibody (middle panel) or immunoprecipitates were probed with anti-Flag antibody (bottom panel). This figure represents the work of J. Knez, except the Western blots shown in the middle and bottom panels.

domain, in an HBM-dependent manner, and the HCF-1 basic domain interacts with a region of E2F-4 separate from the HBM.

3.3.5 P134S mutation in the context of the kelch domain abrogates binding to E2F-4

The HCF-1 kelch domain has been shown to interact with a number of cellular proteins that contain an HBM (Hayes and O'Hare, 1993; Freiman and Herr, 1997; Lu *et al.*, 1998; Lu and Misra, 2000a; Lin *et al.*, 2002; Mahajan *et al.*, 2002a; Luciano and Wilson, 2003; Wysocka *et al.*, 2003). Mutation of the HBM is sufficient to abrogate binding to these proteins. Conversely, a P134S mutation within the kelch domain, which also results in cell cycle arrest in tsBN67$_{HR1}$ cells, is sufficient to abrogate binding to HBM containing proteins (Freiman and Herr, 1997; Lu and Misra, 2000a; Mahajan *et al.*, 2002a), but not to proteins which bind to other regions of HCF-1 (section 3.1.9). We therefore asked whether a P134S mutation in the context of the kelch domain alone would abrogate binding to E2F-4. J. Knez carried out co-immunoprecipitation experiments with tsBN67$_{HR1}$ cells co-transfected with expression vectors for HA-E2F-4 and Flag-tagged HCF-1$_{1-380}$. As shown in figure 3.25, E2F-4 was present in immune complexes precipitated with anti-Flag from cells that were transfected with both Flag-HCF-1$_{1-380}$ and HA-E2F-4, but not from cells transfected with Flag-HCF-1$_{1-380P134S}$ and E2F-4. These results support earlier findings showing an interaction between the HCF-1 kelch domain and E2F-4 in mammalian cells and indicate that the P134S mutation, in the context of the kelch domain, abrogates binding to E2F-4, suggesting that E2F-4 may play a role in cell cycle progression mediated by HCF-1.

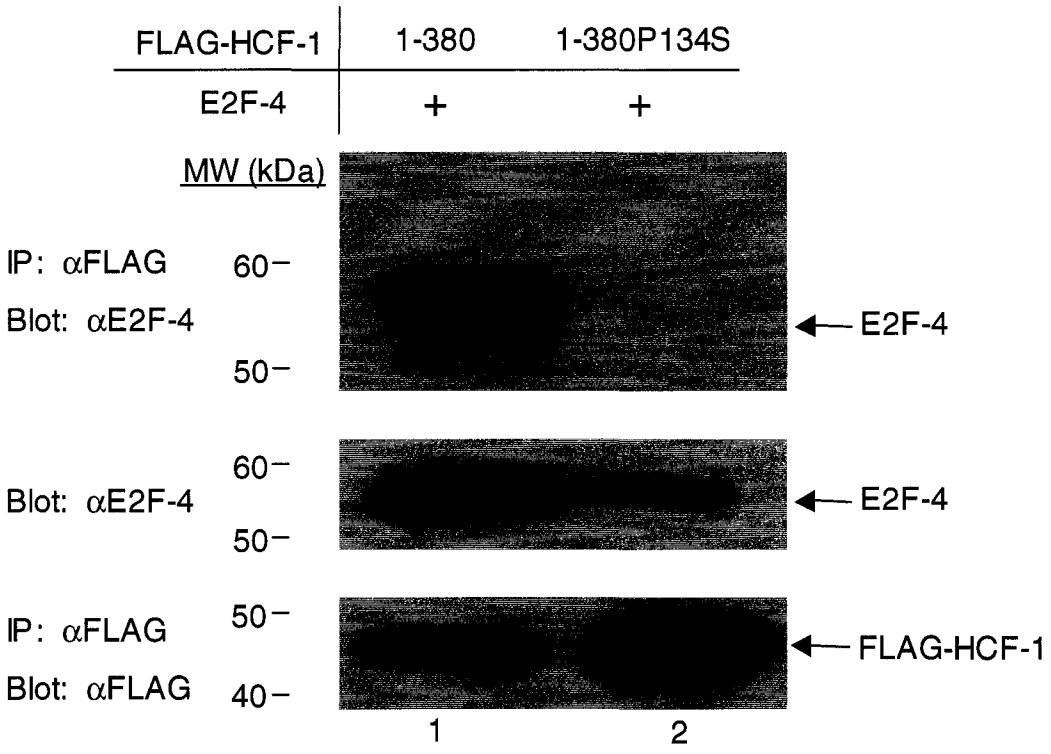


Figure 3.25: P134S mutation, in the context of the kelch domain, abrogates binding to E2F-4. tsBN67$_{HR1}$ cells were transfected with expression vectors for Flag-HCF-1$_{1-380}$ or Flag-HCF-1$_{1-380P134S}$, along with wild type E2F-4,as indicated. Cell extracts were immunoprecipitated (IP) with anti-Flag antibody and the precipitates were resolved on a SDS-polyacrylamide gel and probed with anti-E2F-4 antibody to detect HA-E2F-4 (top panel). To monitor protein expression, extracts were blotted directly using anti-E2F-4 antibody (middle panel) or immunoprecipitates were probed with anti-Flag antibody (bottom panel). This figure represents the work of J. Knez, except the Western blotting in the middle and bottom panels.

3.3.6 An HBM mutation in E2F-4 abrogates an *in vitro* interaction with HCF-1

We performed additional experiments in order to confirm an interaction between HCF-1 and E2F-4 using *in vitro* GST pull-down assays with radiolabeled proteins synthesized *in vitro*. As shown in figure 3.26, in experiments performed by J. Pontoriero, radiolabeled HCF-$1_{1\text{-}902}$ interacts with a fragment of E2F-4, spanning the carboxy-terminal 70 amino acids fused to GST (GST-E2F-4_{C70}), containing the HBM, similar to its interaction with GST-VP16, but not with GST alone. To confirm that E2F-4 contains a functional and *bona fide* HBM we also tested interaction between radiolabeled HCF-$1_{1\text{-}902}$ and an HBM mutant version of the E2F-4 carboxy-terminal fragment fused to GST. In contrast to binding the wild type GST-E2F-4_{C70}, HCF-$1_{1\text{-}902}$ failed to interact with the HBM mutant version, GST-E2F-$4_{C70(Y392A)}$. To ensure that this fragment of E2F-4 remained functional, we have also shown that the interaction between E2F-4 and the pocket protein, p130, is unaffected by the Y392A mutation in E2F-4, even though the HBM is adjacent to the pocket protein binding motif in E2F-4. Thus, radiolabelled p130 bound equally efficiently to both GST-E2F-4_{C70} and GST-E2F-$4_{C70(Y392A)}$ but failed to interact with GST alone. These results confirm the interaction between HCF-1 and E2F-4 and further prove that E2F-4 contains a *bona fide* HBM.

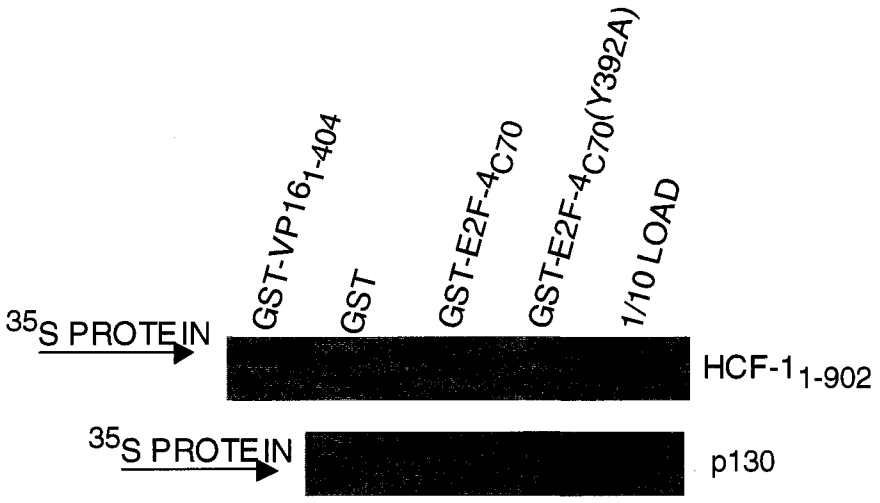


Figure 3.26: An HBM mutation in E2F-4 abrogates an *in vitro* interaction with HCF-1. An *in vitro* GST pull-down assay was performed to detect a direct interaction between HCF-1 and E2F-4. ^{35}S-methionine labeled HCF-1$_{1-902}$ and p130 synthesized *in vitro* were incubated with GST alone or with various GST fusion proteins as indicated and bound material was analyzed by SDS-PAGE. The 1/10 load lane represents 1/10 of the ^{35}S-methionine labeled protein added to the respective binding assay. This figure represents the work of J. Pontoriero.

3.3.7 E2F-4 interferes with HCF-1-mediated cell cycle rescue of the tsBN67$_{HR1}$ cell cycle progression defect

Finally, we sought to determine a biological significance to the HCF-1/E2F-4 interaction using an assay for HCF-1 function involving *in vivo* complementation of tsBN67$_{HR1}$ cells (Wilson *et al.*, 1997; Scarr and Sharp, 2002). To determine if E2F-4 is involved in HCF-1-mediated cellular proliferation in the tsBN67$_{HR1}$ cell line, the effect of co-transfection with E2F-4 on complementation by HCF-1 was determined. tsBN67$_{HR1}$ cells were transfected with pSV2-NEO, or HCF-1$_{1-1011}$, in combination with wild type E2F-4, E2F-4$_{Y392A}$, or the E2F-4 empty vector as shown in figure 3.27. Interestingly, co-transfection of either E2F-4$_{WT}$ or E2F-4$_{Y392A}$ in conjunction with HCF-1$_{1-1011}$ greatly decreased colony formation (compare plate d with f and h) suggesting that overexpression of E2F-4 can interfere with HCF-1 function in cellular proliferation. No appreciable difference is seen in the ability of mutant (Y392A) versus wild type E2F-4 to interfere with HCF-1 function in cellular proliferation (compare plates f and h versus d), most likely due to the fact that HCF-1 was shown to bind both wild type E2F-4 and E2F-4$_{Y392A}$ equally effectively (see figure 3.23; compare lanes 3 and 4). These results indicate that the interaction between HCF-1 and E2F-4 may suppress HCF-1 function in stimulating cellular proliferation. A Western blot is shown in figure 3.27b demonstrating that both wild type E2F-4 and E2F-4$_{Y392A}$ were expressed to comparable levels in tsBN67$_{HR1}$ cells following transfection.

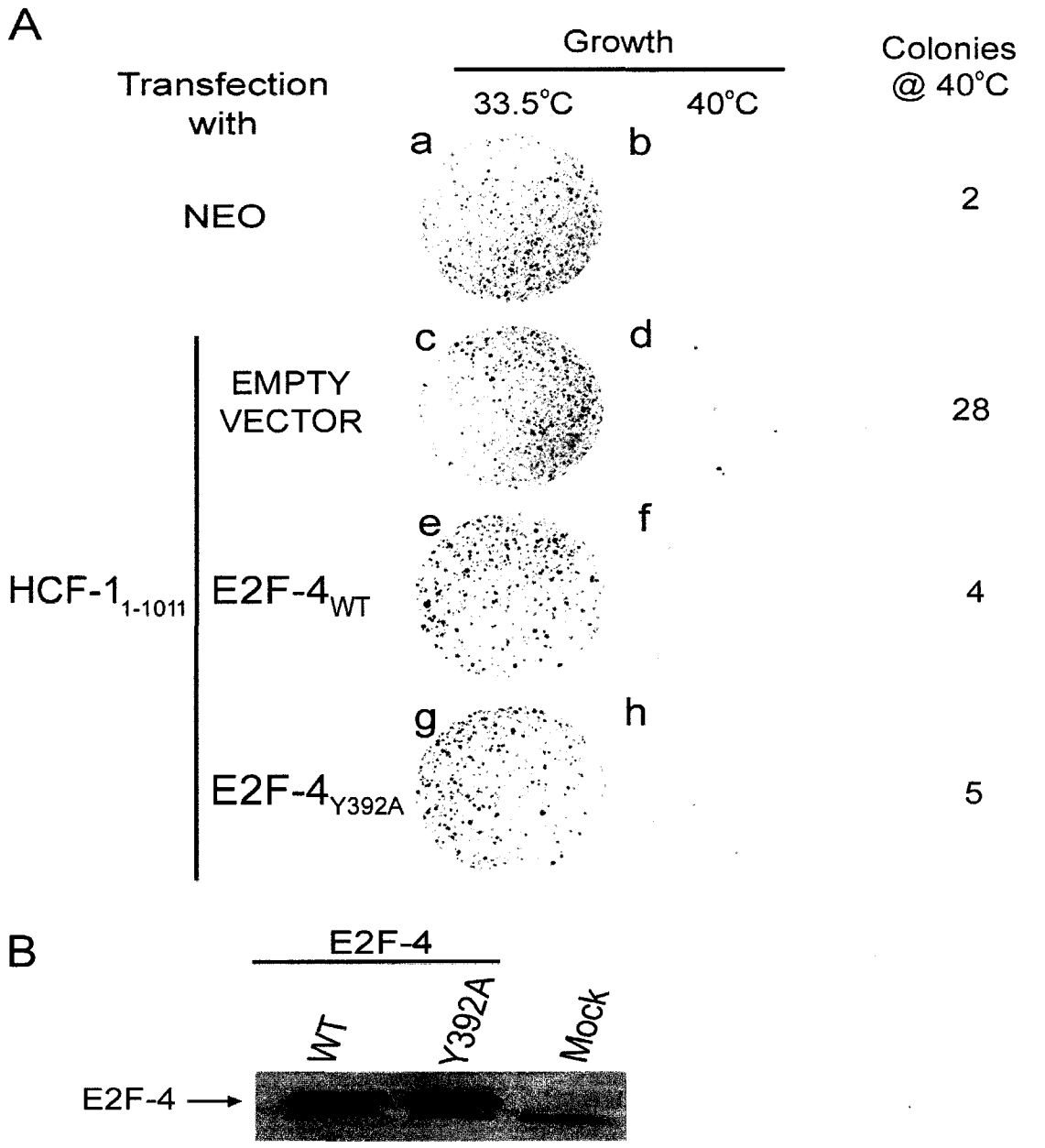


Figure 3.27: E2F-4 interferes with HCF-1-mediated cell cycle rescue of the temperature-sensitive tsBN67$_{HR1}$ cellular proliferation defect. A. tsBN67$_{HR1}$ cells were transfected with pSV2-NEO, or HCF-1$_{1-1011}$, in conjunction with wild type HA-E2F-4, HA-E2F-4$_{Y392A}$, or empty vector, as shown. Cells were grown at 33.5°C or 40°C under G418 selection for 3 weeks and visualized by staining with crystal violet. Colonies @ 40°C represents the number of colonies visible on the plate following 3 weeks of growth from the representative experiment. B. The expression levels of HA-E2F-4$_{WT}$ and HA-E2F-4$_{Y392A}$ were determined by Western blotting with anti-HA antibody. Lysates were prepared from tsBN67$_{HR1}$ cells transfected with 1.0μg of the expression vector for the indicated E2F-4 constructs. The results show representative plates from an experiment performed three times.

CHAPTER FOUR

DISCUSSION

HCF-1 is a ubiquitously expressed and evolutionarily conserved protein (Frattini *et al.*, 1994; Kristie *et al.*, 1995; Wilson *et al.*, 1995b; Kristie, 1997; Liu *et al.*, 1999; Mahajan *et al.*, 2002b) that is essential for normal cell cycle progression through the G1/S transition (Goto *et al.*, 1997; Wilson *et al.*, 1997; Julien and Herr, 2003). However, despite a rapidly expanding list of HCF-1 interaction partners, our understanding of the mechanisms of action of HCF-1 is incomplete. In addition to the importance of the kelch domain, the HCF-1 basic domain is also required to complement the temperature-sensitive cellular proliferation defect in tsBN67 cells, suggesting cooperativity between the kelch and basic domains in HCF-1-mediated cellular proliferation and the possibility that the basic domain also interacts with key cell cycle regulatory proteins (Wilson *et al.*, 1997). We sought to identify novel interaction partners of HCF-1 that may shed light on the mechanisms of HCF-1-mediated cell cycle progression.

The experiments we present here describe novel interactions between HCF-1 and Miz-1 and E2F-4, and a role for HCF-2 in modulating HCF-1 activity. First, the HCF-1/E2F-4 interaction was discovered through a database search for cellular proteins containing the HBM. Interestingly, both E2F-1 and –4, but not other E2Fs, contain an HBM. E2F-4 physically and functionally interacts with both the kelch and basic domains of HCF-1. We show that E2F-4 antagonizes rescue of the tsBN67 temperature-sensitive defect by HCF-1. Second, the HCF-1/Miz-1 interaction was discovered through yeast 2-hybrid screening for HCF-1 basic domain interaction partners. HCF-1 bound within the

Miz-1 transactivation domain resulting in suppression of Miz-1-dependent expression of $p15^{INK4b}$, a cyclin-dependent kinase inhibitor, suggesting a mechanism for HCF-1-mediated cell cycle progression. Third, HCF-2 was found to interact with two HCF-1 basic domain interaction partners, Miz-1 and GABP, and a small carboxy-terminal fragment of HCF-2 which selectively interacted with GABP, mimicked the ability of full length HCF-2 to prevent HCF-1-mediated rescue of the temperature-sensitive cellular proliferation defect in tsBN67 cells. In agreement with this, we demonstrated that an HCF-1 mutant impaired in its ability to bind GABP was also impaired in its ability to rescue the temperature-sensitive defect in tsBN67 cells. Together, these results indicate a role for GABP in this process. Thus, taken together, our findings reveal key cell cycle regulators as novel HCF-1 interaction partners and identify pathways by which HCF-1 may contribute to cellular proliferation.

4.1 IDENTIFYING BASIC DOMAIN INTERACTING PROTEINS

Our findings that HCF-1 physically interacts with Miz-1, a cell cycle regulator that causes cell cycle arrest at G1 (Peukert *et al.*, 1997; Staller *et al.*, 2001; Herold *et al.*, 2002; Zhao *et al.*, 2004), and antagonizes Miz-1-dependent activation of $p15^{INK4b}$, a key cell cycle regulatory protein, provides a provocative new dimension to the activities of these two proteins that may be of consequence to their respective roles in transcription, and their reciprocal effects on cell cycle progression.

4.1.1 HCF-1 may promote cellular proliferation by suppressing Miz-1-dependent expression of $p15^{INK4b}$

Miz-1 is an integral part of the anti-mitogenic TGF-β signaling pathway and contributes to cell cycle arrest following DNA damage and differentiation signals

(Seoane *et al.*, 2001; Herold *et al.*, 2002; Seoane *et al.*, 2002; Wu *et al.*, 2003). Overexpression of Miz-1 results in G1 arrest via a process mediated in part by the induced expression of genes encoding cell cycle inhibitory proteins. Specifically, $p15^{INK4b}$ (Seoane *et al.*, 2001; Staller *et al.*, 2001), $p21^{Cip1}$ (Seoane *et al.*, 2002; van de Wetering *et al.*, 2002; Wu *et al.*, 2003), $p57^{Kip2}$ (Adhikary *et al.*, 2003), cyclin-dependent kinase inhibitors, and Mad4 (Kime and Wright, 2003), a myc antagonist, contain DNA elements within their core promoters which recruit Miz-1 and allow Miz-1-dependent transcriptional activation. Myc interacts with Miz-1 and suppresses Miz-1-dependent transcriptional activation of these genes in the absence of TGF-β or DNA damage and differentiation signals (Seoane *et al.*, 2001; Herold *et al.*, 2002; Wu *et al.*, 2003), by preventing the recruitment of the co-activator, p300, consequently relieving cell cycle arrest and allowing cellular proliferation or apoptosis (Staller *et al.*, 2001; Seoane *et al.*, 2002). Conversely, growth arrest signals relieve myc-mediated repression by causing the dissociation of myc from Miz-1 and allowing expression of cell cycle inhibitory proteins, like $p15^{INK4b}$ (Amati, 2001). Importantly, constructs of myc that are unable to bind to Miz-1 fail to inhibit accumulation of $p15^{INK4b}$ mRNA and are deficient in immortalization (Staller *et al.*, 2001). Thus, growth inhibition pathways stimulated by TGF-β and growth stimulation pathways induced by c-myc converge through Miz-1 on the regulation of $p15^{INK4b}$ gene transcription (Amati, 2001; Gartel and Shchors, 2003; Wanzel *et al.*, 2003). Interestingly, $p15^{INK4b}$ null mice develop tumours and loss of $p15^{INK4b}$ expression due to promoter methylation has frequently been shown in hematopoietic malignancies such as leukemias and lymphomas (Bastova *et al.*, 1997; Herman *et al.*, 1997; Malumbres *et al.*,

1997; Latres *et al.*, 2000). In addition, although mice lacking $p21^{Cip1}$ do not display a predisposition to cancer development, MEFs lacking $p21^{Cip1}$ are defective in the DNA damage G1 checkpoint and loss of $p21^{Cip1}$ functionally cooperates with Ras transformation in the induction of aggressive tumours *in vivo* (Deng *et al.*, 1995; Missero *et al.*, 1996). $p57^{Kip2}$ null mice have altered differentiation and proliferation and demonstrate an increase in apoptotic cells (Yan *et al.*, 1997; Zhang *et al.*, 1997). These mice display renal medullary dysplasia, adrenal cortical hyperplasia and lens cell hyperproliferation and apoptosis (Zhang *et al.*, 1997). These observations suggest that altering the expression of Miz-1 target genes, including $p15^{INK4b}$, may contribute to tumourigenesis.

Functional analysis indicated that HCF-1 represses transactivation by Miz-1 both on the natural $p15^{INK4b}$ promoter and on artificial GAL4 responsive promoters, in a manner that was associated with recruitment of p300 to Miz-1. This may point to a potential mechanism by which HCF-1 may stimulate cell cycle progression. Thus, as observed with c-myc (Staller *et al.*, 2001), inhibition of Miz-1 activation of $p15^{INK4b}$ by HCF-1 may be expected to result in increased activity of cyclinD1/CDK4 and, thus, progression through the G1/S restriction point. Interestingly, repression of $p15^{INK4b}$ expression was potentiated in the presence of both HCF-1 and c-myc, perhaps reflecting an aggregate response since both c-myc and HCF-1 inhibit recruitment of p300 to Miz-1 (Staller *et al.*, 2001). This suggests that these proteins may function in a cooperative manner to stringently control expression of this pivotal cell cycle regulatory target gene.

4.1.2 HCF-1 targets the Miz-1 POZ and transactivation domains: possible mechanisms of interference with Miz-1 activity

HCF-1 targets two independent regions of Miz-1; the amino-terminal POZ domain, and the carboxy-terminal region spanning residues 637-803. The latter region harbours overlapping but distinguishable determinants for interaction with c-myc and the co-activator p300 (Peukert *et al.*, 1997; Staller *et al.*, 2001), while the POZ domain associates with TopBP1 (Herold *et al.*, 2002), suggesting the possibility of functional interplay among these factors in their interactions with Miz-1. Interestingly, we demonstrated that the 637-803 region functions as a potent autonomous transactivation domain and analysis indicates that the determinants required for binding to HCF-1 and for transactivation overlap. Thus, as in several of its interaction partners (Luciano and Wilson, 2002; Vogel and Kristie, 2000a; Luciano and Wilson, 2003), HCF-1 targets a transactivation function in Miz-1. This underscores a potential general functional property of HCF-1 whereby it modulates the activity of its partner proteins by directly interacting with their respective transactivation domains. Interestingly, transcriptional activation mediated by the isolated carboxy-terminal domain was significantly more robust *vis-à-vis* full length Miz-1 or the ΔPOZ-Miz-1 derivative, suggesting that in the context of the full length protein other regions of Miz-1 lying between residues 109-637 may serve to dampen the activation potential of the carboxy-terminal transactivation domain. The full transcriptional activation potential of Miz-1 may thus only be triggered at times when it has been relieved of this attenuation.

Interference with p300 recruitment is one of several possible mechanisms by which HCF-1 inhibits Miz-1 activity because HCF-1 also independently associates with

the Miz-1 POZ domain. POZ domains are conserved protein-protein interaction motifs found at the amino-terminus of many Zn-finger proteins and a group of Poxvirus proteins, and when found as part of transcription factors usually function as negative regulatory domains (Bardwell and Treisman, 1994; Albagli *et al.*, 1995; Collins *et al.*, 2001), in part through recruitment of co-repressors such as N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptor) (Dhordain *et al.*, 1997; Wong and Privalsky, 1998). The Miz-1 POZ domain is thought to be in a latent repressive state and it has been postulated that c-myc serves to convert the Miz-1 POZ domain into an active repressive domain (Peukert *et al.*, 1997). Our findings demonstrate that HCF-1, as well as the minimal Miz-1 interaction sub-region (residues 750-902 of HCF-1), inhibits transactivation of both full length and ΔPOZ GAL4-Miz-1 fusion proteins to a similar degree, suggesting that the POZ domain is not involved in HCF-1-mediated repression. The relevance of HCF-1 interaction with the Miz-1 POZ domain is unclear at present, however, it may be related to a recent report showing that Miz-1 is present in the cytoplasm in association with microtubules (Ziegelbauer *et al.*, 2001). In the presence of drugs that induce expression of the low density lipoprotein receptor (LDLR), Miz-1 translocates to the nucleus where it binds to, and activates transcription of the LDLR gene (Ziegelbauer *et al.*, 2001). Translocation under these circumstances required the integrity of the POZ domain, suggesting that the POZ domain may be required for nuclear import of Miz-1 under certain conditions (Ziegelbauer *et al.*, 2001). HCF-1 has been shown to serve as a nuclear import factor for VP16 (LaBoissiere *et al.*, 1999), and thus could potentially play a similar role with Miz-

1. Native HCF-1 is found almost exclusively in the nucleus (Kristie *et al.*, 1995; Wilson *et al.*, 1995a; LaBoissiere *et al.*, 1999; Wysocka *et al.*, 2001; Izeta *et al.*, 2003b); however, discrete amino-terminal sub-fragments of HCF-1 have been shown to accumulate in the cytoplasm at G0 (Scarr *et al.*, 2000). It is interesting to speculate that these sub-fragments may modulate nuclear translocation of Miz-1, thereby potentiating the repressive effects on Miz-1 activity. In addition, the relevance of the HCF-1/Miz-1 POZ domain interaction may involve regulating the interaction of Miz-1 with TopBP1, another cellular factor shown to associate with the Miz-1 POZ domain (Herold *et al.*, 2002). TopBP1, a cellular protein involved in the cellular response to DNA damage, associates with Miz-1 on the $p15^{INK4b}$ promoter and inhibits transactivation by Miz-1 (Herold *et al.*, 2002). However, upon UV irradiation, TopBP1 is released from Miz-1, freeing active Miz-1, which can activate transcription (Herold *et al.*, 2002). Perhaps, HCF-1 and TopBP1 act cooperatively in regulating Miz-1 activity in the absence of UV irradiation.

4.1.3 HCF-1 possesses the characteristics of a transcriptional co-regulatory factor

We have identified Miz-1 as a novel interaction partner for HCF-1 and illustrate an intriguing new pathway of regulation that potentially links the opposing effects of these two proteins on cell cycle control. Our findings also represent the first example of HCF-1 inhibiting transactivation of an associated transcription factor. Subsequent evidence has been provided by other groups indicating a dual function for HCF-1 in transcriptional control whereby depending on the context, it is expected that HCF-1 will exhibit either co-activation or co-repression properties (Wysocka *et al.*, 2003). Several

studies have shown that HCF-1 interacts with the transcriptional activation domain of its interaction partners and contributes to activation (Luciano and Wilson, 2000; Vogel and Kristie, 2000a; Luciano and Wilson, 2003), often contributing its own autonomous transcriptional activation domain (Luciano and Wilson, 2000; Luciano and Wilson, 2003). However, recent reports support a role for HCF-1 interacting either directly (Wysocka *et al.*, 2003) or indirectly (Scarr and Sharp, 2002) with histone deacetylase transcriptional co-repressors and potentially inhibiting transcription activation in certain contexts due to its affects on chromatin structure. Furthermore, Herr and colleagues demonstrated that the ability of HCF-1 to recruit either transcriptional co-activators, like histone methyltransferases, or transcriptional co-repressors, like histone deacetylases, is dependent on its interaction partners, at least in the case with VP16 (Wysocka *et al.*, 2003). Thus, HCF-1 molecules bound to VP16 are selectively bound to histone methyltransferase in the absence of repressive histone deacetylases, thus allowing for transcriptional co-activation of HSV IE genes in this context (Wysocka *et al.*, 2003).

This evidence suggests that HCF-1 shares with several other transcription factors the ability to regulate transcription either positively or negatively. For instance, both c-myc and SMADs are transcription factors with well-defined autonomous transcription activation domains which have been shown to be recruited to DNA by sequence-specific sites in several gene promoters and activate transcription (Dang, 1999; Dijke *et al.*, 2002; Gartel and Shchors, 2003; Shi and Massague, 2003; Wanzel *et al.*, 2003). However, in other cases these proteins are recruited to gene promoters and serve to repress transcription (Dang, 1999; Dijke *et al.*, 2002; Gartel and Shchors, 2003; Shi and

Massague, 2003; Wanzel *et al.*, 2003). The transcriptional outcome appears to depend on the context, specifically on the organization of elements within the promoter and protein-protein interactions (Dang, 1999; Dijke *et al.*, 2002; Gartel and Shchors, 2003; Shi and Massague, 2003; Wanzel *et al.*, 2003; Frederick *et al.*, 2004). Furthermore, a novel HCF-1 related protein called HCLP-1 has been identified and shown to inhibit transactivation by LZIP (Zhou *et al.*, 2001). Thus, HCF-1 and its family members may function as both co-activators and co-repressors of transcription. We believe that this dual nature may be utilized by HCF-1 in cell cycle control since HCF-1 could function as a co-activator of genes which are required for cell cycle progression while also functioning as a co-repressor of genes, such as $p15^{INK4b}$, which serve to inhibit cell cycle progression.

4.1.4 Miz-1 may be one of several effectors in HCF-1-mediated cellular proliferation

The discovery of Miz-1 as an HCF-1 interaction partner is intriguing given its role in cell cycle arrest at G1 and since HCF-1 was shown to antagonize its ability to upregulate expression of $p15^{INK4b}$. Miz-1 targets determinants present in the basic region of HCF-1, a domain that in conjunction with the amino-terminal region, is able to rescue the temperature-sensitive proliferation defect in tsBN67 cells (Wilson *et al.*, 1997). Whether Miz-1 is an important effector in this process remains to be determined. Herr and co-workers demonstrated that the minimal fragment of HCF-1 capable of rescuing the cell cycle defect in tsBN67 cells was residues 1-902, and that further carboxy-terminal deletion of this fragment to residue 836 abrogated rescue (Wilson *et al.*, 1997). The Miz-1 minimal binding interface mapped to residues 770-836, and while residues between 836 and 902 enhanced binding with Miz-1, they are neither necessary nor

sufficient for interaction. Moreover, the causative mutation (P134S) in HCF-1 leading to cell cycle arrest does not affect interaction with, or transactivation by, Miz-1. Furthermore, a variety of experiments presented in this thesis indicate that Miz-1 does not play a role in HCF-1-mediated cellular proliferation in tsBN67 cells. For instance, co-expression of Miz-1 did not interfere with HCF-1-mediated rescue of tsBN67 cells. Furthermore, a small fragment of HCF-1 specific for Miz-1 binding did not affect HCF-1-mediated rescue of tsBN67 cellular proliferation. There may be a number of possibilities as to why Miz-1 does not affect HCF-1-mediated cellular proliferation in tsBN67 cells. First, perhaps the Miz-1/HCF-1 interaction does not play a role in rapidly dividing cells; instead this interaction may be important in cell cycle control at very specific times such as embryogenesis/development or in specific cells. Interestingly, Miz-1 null embryos are not viable, indicating that Miz-1 is required for embryonic development (Adhikary *et al.*, 2003). Perhaps, the Miz-1/HCF-1 interaction is also required during embryogenesis. In addition, Miz-1 may not affect HCF-1-mediated tsBN67 cellular proliferation because HCF-1 is dominant to Miz-1 in these cells. A similar example is seen with the retinoblastoma protein and viral oncoproteins in HeLa cells where wild type pRb does not cause cell cycle arrest whereas mutants of pRb unable to bind to viral oncoproteins do cause cell cycle arrest (Dick and Dyson, 2002). Finally, tsBN67 cells were initially created through a chemical mutagenesis protocol which resulted in the HCF-1 mutation and the consequent temperature-sensitive phenotype (Goto *et al.*, 1997). There is evidence that this cell line may have received additional mutations (LaBoissiere and O'Hare, 2000). Therefore, it is possible that Miz-1 is

ineffective in tsBN67 cells due to a mutation(s) in downstream effectors of the Miz-1 pathway. In any case, our results suggest that the Miz-1/HCF-1 interaction is not critical in HCF-1-mediated rescue of the tsBN67 temperature-sensitive cellular proliferation defect, but may play a role in specific cells and/or at specific times in embryogenesis or development. The foregoing implies that Miz-1 may not be necessary for HCF-1-mediated cell cycle control and that the phenotypic basis of the P134S mutation may be related to as yet unidentified proteins that target the amino terminal and/or basic regions. However, this does not necessarily negate a role for Miz-1 since cell cycle control by HCF-1 is likely a highly complex process that involves the cooperative interplay with many effector targets that recognize both the amino-terminal kelch domain and the adjacent basic region. Thus, Miz-1 interaction may prove to be necessary in HCF-1-mediated cell cycle progression thus explaining the requirement of the HCF-1 basic domain in the rescue of the temperature-sensitive proliferation defect in tsBN67 cells.

4.1.5 A role for GABP in HCF-1-mediated cellular proliferation

In contrast to Miz-1, various experiments presented in this thesis suggest that the GABP/HCF-1 interaction is important in HCF-1-mediated rescue of tsBN67 cellular proliferation. For instance, a triple alanine substitution at HCF-1 amino acids 793-795 reduced binding to GABP by 3-fold as determined by a quantitative β-galactosidase activity assay. This mutation also compromised the ability of HCF-1 to rescue the tsBN67 cellular proliferation defect. In addition, a fragment of HCF-2 which only bound GABP diminished rescue of the tsBN67 cellular proliferation defect by HCF-1 thus functioning as a trans-dominant inhibitor in this assay. HCF-1 has been shown to

function as a co-activator of GABP (Vogel and Kristie, 2000a). Given the fact that GABP has been shown to be involved in the transcriptional activation of several growth-promoting genes (Scott *et al.*, 1994; Imaki *et al.*, 2003) we propose that the GABP/HCF-1 interaction is important in HCF-1-mediated cellular proliferation since HCF-1 must contribute to GABP-directed activation of required growth-promoting genes. The fact that HCF-2 inhibits HCF-1-mediated rescue of tsBN67 cellular proliferation and binds GABP further supports a role for a GABP/HCF-1 interaction in this process.

4.1.6 Basic domain interaction partners are also targeted by HCF-2

Our findings that HCF-2 shares common interaction partners with HCF-1 provides an additional commonality between these related proteins. Although these proteins share much in common, they have been shown to be functionally antagonistic in their roles in cellular proliferation (Johnson *et al.*, 1999) and HSV gene expression (Lee and Herr, 2001). Our finding that an HCF-2 fragment which selectively interacts with GABP retains the ability of full length HCF-2 to inhibit HCF-1-mediated rescue of the temperature-sensitive defect in tsBN67 cells provides insight into HCF-2's role in cell cycle control and suggests that the shared interaction with GABP may contribute to the antagonistic roles HCF-1 and HCF-2 play in cellular proliferation. HCF-2 shares homology with the carboxy-terminal domain of HCF-1, known as the Fn3 repeats, which fold into two β sheets packed against each other to form an extended groove that can form an attractive docking site for interacting proteins (Wilson *et al.*, 2000). The repeats function as a self-association domain in HCF-1 in which association between the carboxy-terminal Fn3 repeats and amino-terminal residues 360-402 allows for non-

covalent association of amino- and carboxy-terminal fragments following proteolytic cleavage of the HCF-1 precursor polypeptide (Wilson *et al.*, 2000). HCF-2 lacks the HCF_{PRO} repeats and is not proteolytically processed (Johnson *et al.*, 1999) therefore, it is surprising that the SAS1 elements of HCF-1 are conserved in HCF-2 and ceHCF (Wilson *et al.*, 2000). Herr and co-workers (Wilson *et al.*, 2000) suggested that the presence of SAS1 sequences in ceHCF and HCF-2 may indicate a dual role for these sequences in HCF-1 function that is conserved in ceHCF and HCF-2. Thus, these sequences (SAS1) are conserved in ceHCF and HCF-2, not for self-association, but presumably for protein-protein interactions with shared cellular factors (Wilson *et al.*, 2000). Consistent with the hypothesis that the Fn3 repeats may interact with cellular proteins in addition to functioning as self-association sequences, we found that this domain interacts with Miz-1 and GABP. Although it has been anticipated that the Fn3 repeats of HCF-2 may serve as a protein-protein interaction surface and that HCF-1 and HCF-2 share common interaction partners (Johnson *et al.*, 1999; Wilson *et al.*, 2000), it was not expected that the HCF-2 Fn3 repeats would bind to proteins that bound the HCF-1 basic domain. Interestingly, although GABP was found to initially interact with the HCF-1 basic domain (Vogel and Kristie, 2000a), we have shown that the HCF-1 Fn3 repeats are also sufficient to interact with GABP in a yeast 2-hybrid assay (data not shown). However, the reason why Miz-1 is able to interact with the basic domain in HCF-1 and the Fn3 repeats in HCF-2 is not known. Perhaps Miz-1 contains separate regions which mediate binding to these distinct domains or perhaps the HCF-1 basic domain shares with the HCF-2 Fn3 repeats a small motif responsible for Miz-1 binding. In any case, we have

identified Miz-1 and GABP to be common interaction partners for HCF-1 and HCF-2. Moreover, a fragment sufficient to bind GABP retained the ability of full length HCF-2 to interfere with HCF-1-mediated rescue, demonstrating that this shared interaction contributes to the ability of HCF-2 to prevent complementation of the temperature-sensitive defect in tsBN67 cells. Thus, HCF-2 may sequester GABP from HCF-1, preventing a critical function required for cellular proliferation. HCF-2/GABP interaction likely will not mimic the function of HCF-1/GABP interaction since HCF-2 has different properties and displays different functions than HCF-1 (Johnson *et al.*, 1999; Lee and Herr, 2001). For instance, both HCF-1 and HCF-2 can form a VP16-induced complex on IE gene promoters, however, only HCF-1 results in transcriptional activation of these genes (Lee and Herr, 2001). In addition, whereas HCF-1 is predominantly nuclear (Kristie *et al.*, 1995; Wilson *et al.*, 1995a; LaBoissiere *et al.*, 1999; Wysocka *et al.*, 2001; Izeta *et al.*, 2003b), HCF-2 can be both nuclear and cytosolic and may shuttle between compartments in a cell cycle-dependent manner (Johnson *et al.*, 1999). Thus, HCF-2 may bind GABP, but lack the co-activation function, thus preventing expression of key GABP/HCF-1-growth-promoting genes. In addition, HCF-2 may re-localize GABP to the cytosol, again preventing expression of key GABP/HCF-1-growth-promoting genes. In summary, we have shown that HCF-2 shares interaction partners with HCF-1, and through its interaction with GABP may antagonize HCF-1-mediated cellular proliferation.

4.2 IDENTIFYING KELCH DOMAIN INTERACTING PROTEINS

Our findings that E2F-4, an integral regulator of cell cycle control, physically interacts with HCF-1 and suppresses HCF-1-mediated cellular proliferation, provides insight into the cellular function of HCF-1 and expands the list of its interaction partners that may be of consequence to its role in cell cyle control.

4.2.1 E2F-4 targets the HCF-1 kelch and basic domains

Since all known kelch binding proteins contain an HBM, a database search was conducted for cellular proteins that contain an HBM. This search has revealed that 2 members of the E2F family of transcription factors, E2F-1 and E2F-4 contain the HBM. Given the central role played by E2F factors in cell cycle control (DeGregori, 2002; Trimarchi and Lees, 2002), we pursued this finding further. In addition to demonstrating an interaction we found that the HCF-1/E2F-4 association was mediated by determinants in both the HCF-1 kelch and basic domains. Thus, E2F-4 is the first example of an HCF-1 interaction partner that targets both domains. The significance of this is not yet clear, however it may provide a mechanism to explain the apparent paradox of multiple HBM-containing proteins simultaneously associating with HCF-1 (Wysocka *et al.*, 2003). The separate, independent binding sites may accommodate the recruitment of a distinct set of HCF-1 accessory factors under different physiological conditions or in response to different biological cues (Wysocka *et al.*, 2003) that allow for a variety of functions or outcomes. Similarly, the activity of E2F-4 and HCF-1 may be differentially modulated depending on whether E2F-4 is bound to the kelch domain, the basic domain, or both. In addition, this finding may be relevant to previous findings concerning HCF-1-dependent

rescue of the tsBN67 cell cycle progression defect. It has been shown that cell cycle arrest occurs as a result of a mutation in the kelch domain, implying that an interaction with an HBM-containing protein is required (Wilson *et al.*, 1997). However, the kelch domain is not sufficient, as the adjacent basic domain is also required (Wilson *et al.*, 1997). It has been hypothesized that this is due to the fact that cell cycle control by HCF-1 is a highly complex process that involves the cooperative interplay with many effector targets that recognize the kelch and basic domains. However, the findings presented here highlight the possibility that a single factor, in this case E2F-4, may bind simultaneously to both domains, and it is the binding to both domains that is required for cell cycle progression, thus explaining why both domains must be present for HCF-1-mediated cell cycle progression.

Incidentally, our finding that E2F-4 interacts with HCF-1 through its HBM and a distinct second region is not without precedent in the literature. In fact, the ability of two proteins to interact through a conserved motif as well as additional contacts has been demonstrated previously with protein phosphatase PP1c and its targeting subunits which contain a well defined RVxF motif (Cohen, 2002). For example, mutation of the RVxF motif in AKAP220 does not abrogate binding to PP1c (Schillace *et al.*, 2001). Furthermore, an interaction independent of the RVxF motif has been demonstrated between NIPP1 and PP1c (Beullens *et al.*, 2000). These additional contacts not only stabilize binding but are speculated to modulate the activity and/or specificity of PP1c.

4.2.2 Possible mechanisms by which E2F-4 antagonizes HCF-1-mediated cellular proliferation

Interaction of E2F-4 with HCF-1 via the kelch domain is sensitive to the P134S mutation that is responsible for the cell cycle arrest phenotype in tsBN67 cells (Goto *et al.*, 1997), suggesting that E2F-4 may play an important role in this context. However, it is unlikely that E2F-4 on its own serves as a critical regulator of HCF-1-mediated cell cycle control in tsBN67 cells. This is suggested by our findings that the P134S mutation does not abrogate binding to E2F-4 in the context of full length HCF-1 since the loss of binding to the kelch domain is compensated by binding via the basic domain. Nevertheless, given its prominent role in G1 progression, it is probable that E2F-4, in conjunction with other factors, does play an important role in cell cycle control by HCF-1. In fact, we have demonstrated that E2F-4 acts in opposition to HCF-1 in tsBN67 cells. Specifically, the biological outcome of the HCF-1/E2F-4 interaction was to prevent rescue of the tsBN67 cell proliferation defect by HCF-1 in the presence of E2F-4. This, along with other reports demonstrating that HCF-1 interacts with a variety of cellular factors reinforces the hypothesis that cellular proliferation mediated by HCF-1 is due to multiple effectors that bind to both the kelch and basic domains.

E2F-4 is a complex and dynamic protein, therefore, it may prevent rescue by HCF-1 in a number of ways. Since the E2F-4 Y392A mutant retained the ability to repress HCF-1-mediated cell cycle rescue, it is possible that E2F-4 prevents rescue by HCF-1 in a manner that is independent of their interaction. However, we do not favour this possibility and believe that the E2F-4 Y392A mutant retained the repressive phenotype because it retained the ability to bind HCF-1.

One mechanism by which E2F-4 and HCF-1 may antagonize each other's activities is by reciprocally affecting each other's transcriptional abilities. E2F-4 is considered a 'repressive E2F' because it binds to E2F sites in E2F-responsive promoters in quiescent or G0–early G1 cells, in association with pocket proteins and HDAC to negatively regulate transcription of growth-promoting genes (Tommasi and Pfeifer, 1995; Vairo *et al.*, 1995; Li *et al.*, 1997; Iavarone and Massague, 1999; Takahashi *et al.*, 2000). Thus, when overexpressed in tsBN67 cells, E2F-4 and associated histone modifying complexes, may be recruited to HCF-1 responsive promoters in growth promoting genes and prevent expression of genes that are necessary for cellular proliferation. Alternatively, in cellular proliferation, HCF-1 may be recruited to E2F sites through E2F-4, thereby converting E2F repressive complexes to activating complexes leading to increased expression of growth promoting factors, including cdc25A and c-myc at the appropriate time (Iavarone and Massague, 1997; Li *et al.*, 1997; Iavarone and Massague, 1999; Chen *et al.*, 2002; Frederick *et al.*, 2004; Yoshida and Inoue, 2004). Thus, although E2F-4 is considered a 'repressive E2F', it contains an autonomous transactivation domain in its C-terminus that binds the acetyltransferase GCN5 and cofactor TRAPP *in vivo* (Lang *et al.*, 2001), and thus may stimulate transcription in a restricted developmental or cell cycle window at certain target promoters. Interestingly, the HBM of E2F-4 is embedded within the E2F-4 transactivation domain (Lang *et al.*, 2001), and a recent report has demonstrated that HCF-1 augments the activity of the E2F-4 activation domain (Luciano and Wilson, 2003). The mechanism by which this occurs is not clear but since the HBM is adjacent to the pocket protein binding site in the E2F-4

transactivation domain (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994), it seems possible that HCF-1 may promote E2F-4 activity by displacing the pocket protein repressor complex (Luciano and Wilson, 2003). Alternatively, HCF-1 may also provide a transactivation function to E2F-4 complexes through its own carboxy-terminal transactivation domain (Luciano and Wilson, 2002).

In addition, E2F-4 could de-regulate HCF-1 activity through altering its subcellular localization. E2F-4 contains 2 closely spaced NES which result in a dynamic subcellular localization throughout the cell cycle (Lindeman *et al.*, 1997; Verona *et al.*, 1997; Gaubatz *et al.*, 2001). In quiescent or G0–early G1 cells, E2F-4 is associated with pocket proteins and localized to the nucleus (Lindeman *et al.*, 1997; Gaubatz *et al.*, 2001). As cells move to mid G1, E2F-4 dissociates from pocket proteins, exposing its NES and translocates to the cyosol (Lindeman *et al.*, 1997; Gaubatz *et al.*, 2001). HCF-1 had been considered to be constitutively nuclear (Kristie *et al.*, 1995; Wilson *et al.*, 1995a; LaBoissiere *et al.*, 1999; Wysocka *et al.*, 2001; Izeta *et al.*, 2003b), but a recent finding illustrates that interaction with HPIP, a protein with an NES, is sufficient to re-locate it to the cytosol (Mahajan *et al.*, 2002a). Therefore, a similar mechanism with E2F-4 at mid G1 in tsBN67 cells could be at play, preventing functions of HCF-1 in the nucleus which are necessary for cellular proliferation.

4.2.3 HCF-1 may regulate 'activating' and 'repressive' E2Fs

E2F-4 was discovered to be an HCF-1 interaction partner based on the presence of an HBM. Interestingly, E2F-1, but not other E2Fs, also contains an HBM. Accordingly, we showed that E2F-1 also interacts with HCF-1. This highlights the

possibility that HCF-1 functions in tandem with both a member of the 'activating E2Fs' as well as a member of the 'repressive E2Fs' to execute cellular proliferation. The recent finding that HCF-1 interacts with both HMT and HDAC proteins underscores the possibility that HCF-1 can broadly control transcriptional outcomes (Wysocka *et al.*, 2003) with E2F and other transcription factors to both activate expression of growth promoting genes while also decreasing expression of growth inhibitory genes. Interestingly, the HBM in E2F-1 is located at the amino-terminus, adjacent to the cyclin A/CDK2 binding site (Krek *et al.*, 1994; Xu *et al.*, 1994). Thus, HCF-1 may positively regulate E2F-1 function through interference with cyclinA/CDK2 binding, thereby preventing phosphorylation of the E2F-1 DNA binding domain, a negative regulatory event which diminishes the affinity of the E2F-1/DP heterodimer for DNA (Krek *et al.*, 1994; Xu *et al.*, 1994). Further studies to elucidate the functional interplay with these, and other, factors involved in cellular proliferation will be of interest.

4.3 CONCLUSIONS

The work presented in this thesis has sought to further characterize the cellular roles of HCF-1 and build on the evidence pointing to a role for HCF-1 in cellular proliferation. We describe two key cell cycle regulatory proteins, Miz-1 and E2F-4, as novel HCF-1 interaction partners, and illustrate how these interactions may mechanistically contribute to HCF-1-mediated cellular proliferation. We have shown that HCF-1 binds to and antagonizes the activity of Miz-1, a transcription factor that induces expression of cyclin-dependent kinase inhibitors that prevent inactivation of pocket proteins, thereby blocking cell cycle progression (Seoane *et al.*, 2001; Staller *et*

al., 2001; Seoane *et al.*, 2002; Adhikary *et al.*, 2003; Wu *et al.*, 2003). We speculate that by antagonizing Miz-1 activity, HCF-1 may indirectly promote pocket protein inactivation and thus cellular proliferation. In addition, we have shown that E2F-4 interacts with both the HCF-1 kelch and basic domains and that overexpression of E2F-4 antagonizes the ability of HCF-1 to promoter cellular proliferation in tsBN67 cells.

In support of the hypothesis that HCF-1 contributes to cell cycle progression by controlling the phosphorylation status of pRb family proteins, Herr and colleagues demonstrated that inactivation of pRb family proteins with SV40 large T antigen and adenovirus E1A can bypass the HCF-1 requirement, and rescue the tsBN67 defect (Reilly *et al.*, 2002) although this is not sufficient to fully overcome a cell cycle block in HCF-1-depleted HeLa cells (Julien and Herr, 2003). Therefore, HCF-1 indirectly promotes cellular proliferation by pRb family protein inactivation, in addition to another distinct function. The research presented here suggests ways in which HCF-1 may control cellular proliferation through pocket protein-dependent and –independent pathways. Specifically, the functional interaction with Miz-1 places HCF-1 upstream of pRb family proteins, whereby HCF-1 controls pRb phosphorylation, and thus, functional status. However, through its interaction with E2F-4, HCF-1 may promote cellular proliferation in a mechanism independent of pRb phosphorylation status. Specifically, recent reports describe how E2F-4/HDAC complexes can act as transducers of TGF-β signals to mediate transcriptional repression of growth promoting genes such as c-myc, cdc25A, E2F-1, B-myb, and HsORC1 (Li *et al.*, 1997; Iavarone and Massague, 1999; Chen *et al.*, 2002). HCF-1 may prevent the formation of repressive E2F-4/HDAC complexes or

convert these to activating complexes. Thus, an E2F-4/HCF-1 complex could provide a mechanism, independent of pRb phosphorylation status, to contribute to cellular proliferation.

Significantly, both Miz-1 and E2F-4 are transducers of TGF-β signals. Upon TGF-β exposure, Miz-1 is relieved of myc-repression and stimulates transcription of growth arrest genes (Amati, 2001), while E2F-4 in association with histone deacetylase complexes represses the transcription of growth promoting genes (Li *et al.*, 1997; Iavarone and Massague, 1999; Chen *et al.*, 2002). Thus, TGF-β causes growth arrest in most cell types by altering the transcriptional profile of both growth promoting and growth arrest genes resulting in hypophosphorylation of pRb which sequesters E2F factors needed for progression into S phase of the cell cycle, thereby leading to cell cycle arrest at G1 (Massague, 2000; Massague *et al.*, 2000; Dijke *et al.*, 2002; Shi and Massague, 2003; Siegel and Massague, 2003). HCF-1 may counteract these effects and promote cellular proliferation through its interactions with Miz-1 and E2F-4. For instance, it is envisioned that HCF-1 induces cell cycle progression by repressing the activation of growth-inhibitory genes ($p15^{INK4b}$, $p21^{Cip1}$, and $p57^{Kip2}$) by interacting with Miz-1, while also activating the expression of growth-promoting genes (c-myc and cdc25A) by interacting with E2F-4 and converting E2F-4 repressive complexes to E2F-4 activating complexes (see figure 4.1).

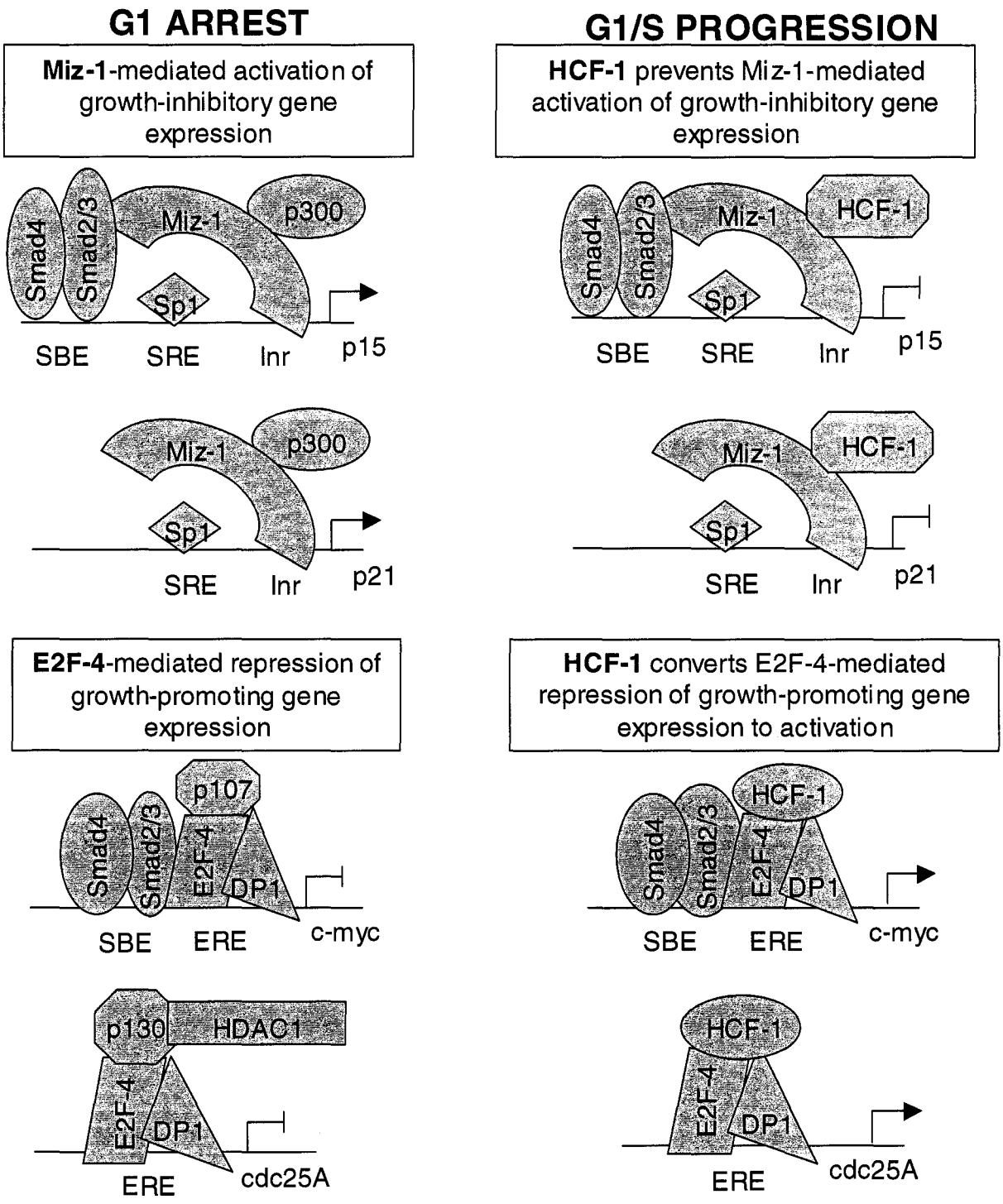


Figure 4.1: A model depicting a mechanism for HCF-1-mediated cellular proliferation. The model describes a mechanism by which HCF-1, through its interactions with Miz-1 and E2F-4, may modulate the levels of expression, through transcriptional control, of both growth-promoting and growth-inhibitory genes. It is proposed that HCF-1 exploits its ability to function as both co-activator and co-repressor to augment expression of growth-promoting genes, while attenuating expression of growth-inhibitory genes.

HCF-1 may promote cellular proliferation and completion of cytokinesis through the association with several interaction partners with effects on multiple independent and synergistic pathways including transcriptional control of gene expression, chromatin association and modification, as well as post-transcriptional effects through control of pre-mRNA processing that are required for cell cycle progression (Wysocka and Herr, 2003). Our identification of Miz-1 and E2F-4, key cell cycle regulatory proteins, as HCF-1 interacting partners provides insight on the mechanism of HCF-1-mediated cellular proliferation and suggests a possible role for HCF-1 in tumour development. Tumour development proceeds via a process in which a succession of genetic changes lead to a progressive conversion of normal cells into cancer cells in which regulatory circuits that govern normal cellular proliferation have become deregulated (Hanahan and Weinberg, 2000; Hahn and Weinberg, 2002). Cancer cells may accumulate defects in regulatory circuits that allow self-sufficiency in growth signals, limitless replicative potential, sustained angiogenesis, and metastasis, and result in insensitivity to growth-inhibitory signals and resistance to apoptosis (Hanahan and Weinberg, 2000; Hahn and Weinberg, 2002). The accumulation of these defects results from the disruption of an anticancer mechanism programmed into cells and are hypothesized to depend on deregulated transcription (Hanahan and Weinberg, 2000; Darnell, 2002; Hahn and Weinberg, 2002). Molecular analysis of human tumours has shown that cell cycle regulators are frequently mutated in cancer which underscores how important the maintenance of cell cycle control is in the prevention of human cancer (Malumbres and Barbacid, 2001). Furthermore, human cancers harbour genomes often containing an

abnormal number of chromosomes, a condition termed aneuploidy (Hahn and Weinberg, 2002; Storchova and Pellman, 2004). Since loss of HCF-1 activity in cells prevents G1/S transition, in addition to a cytokinesis defect which results in polyploidy (Goto *et al.*, 1997; Reilly and Herr, 2002; Julien and Herr, 2003), HCF-1 is emerging as a novel factor which could contribute to the process of cancer development.

Future challenges to unraveling the complex roles of HCF-1 in the cell include ascertaining the interplay of a variety of interactions in HCF-1-mediated cellular proliferation and how these interactions and HCF-1 function are regulated throughout the cell cycle to ensure regulated proliferation. Contemporaneous with our identification and analysis of E2F-4 and other novel HBM-containing HCF-1 interaction partners, Luciano and Wilson published a study identifying a similar list of HBM containing proteins (Luciano and Wilson, 2003). Thus, through these and other studies over the past five years, a large set of cellular proteins have been found that contain the HBM and interact with the HCF-1 kelch domain. However, several important questions remain unanswered. Most importantly, it remains to be determined what role, if any, these proteins play in HCF-1-mediated cell cycle progression. Mahajan and Wilson constructed various mutations within the loops of the HCF-1 kelch domain, some of which abrogated binding to Luman; however, these experiments demonstrated that Luman was neither necessary nor sufficient in HCF-1-mediated cellular proliferation (Mahajan and Wilson, 2000). It will be illuminating to correlate the ability of the mutants to rescue tsBN67 cellular proliferation with their ability to bind the various kelch domain interaction partners. In addition to determining the role played by E2F-4 and

other novel HBM containing partners in HCF-1 function, several other intriguing questions remain. For instance, does HCF-1 interact with the entire set of HBM containing partners simultaneously during G1/S progression, presumably regulating the expression of a large set of target genes, owing to the abundance of HCF-1 in the cell (Wysocka *et al.*, 2001), or rather, are there control mechanisms regulating the binding to HCF-1. For example, are the interactions regulated in a temporal or spatial pattern, and/or is there competition among the various HBM containing partners for HCF-1. Answers to these questions will add to our understanding of the mechanisms by which a large set of HBM containing partners contributes to HCF-1-mediated cellular proliferation.

In addition to numerous kelch domain-interaction partners, the basic domain associates with a variety of cellular proteins in addition to Miz-1. It will be of interest to determine the role played by each of these proteins, and the interplay among them, in HCF-1-mediated cellular proliferation. Linking protein-protein interactions to cell cycle signals that may regulate these interactions at specific phases of the cell cycle also remains to be investigated. For instance, do cell cycle arrest signals, such as TGF-β or DNA damage, cause dissociation of HCF-1 from Miz-1 as a result of post-translational modifications of either, or both, proteins, thereby freeing Miz-1 and allowing cell cycle arrest at appropriate times, as shown to regulate the Miz-1/myc interaction (Seoane *et al.*, 2001; Herold *et al.*, 2002; Wu *et al.*, 2003)? Only through an understanding of the full repertoire of HCF-1 interaction partners, the function and interplay of these interactions, and the signaling pathways which regulate these interactions, will we approach a more

complete appreciation of the complex mechanisms at play in HCF-1-mediated cellular proliferation. Ultimately, the efforts of several research groups will provide us with the answers to several of these questions and propose new avenues of research to unravel the complex roles of HCF-1 in the cell.

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